MICROBIAL ADHESION ON BIOMATERIALS AND THE SOURCES OF HUMAN BETA-DEFENSIN-3 IN SEPTIC JOINT IMPLANT LOOSENING

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Cover photo:
A cross-section of *S.aureus* S-15981 biofilm on a biomaterial surface, viewed with a confocal laser scanning microscope (CLSM) after 48 hours of incubation. In the viability staining, dead bacterial cells are seen in red color and viable cells in green color. The bacteria are embedded in extracellular polymeric substance (EPS).

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ABSTRACT

In the novel diamond-like carbon polymer hybrid (DLC-p-h) coatings, the useful properties of diamond-like carbon (DLC) and a chosen polymer can be combined. So far two unique biomaterials, diamond-like carbon polytetrafluoroethylene hybrid (DLC-PTFE-h) and diamond-like carbon polydimethylsiloxane hybrid (DLC-PDMS-h) with dirt-repelling hydrophobic and oleophobic properties have been developed. These novel coatings were studied in this thesis.

The Diamond Group at the ORTON Research Institute have produced novel biomaterial coatings with the in-house developed Filtered Pulsed Arc Discharge (FPAD)-method. The FPAD method enables the production of thick (>200 µm), high quality (> 80% diamond sp^3 –bonds) DLC coatings with properties needed in high-load bearing applications such as the Total Hip Arthroplasty (THA).

Bacterial adhesion is the initial step in the prosthetic joint infection (PJI) and leads to biofilm formation and the establishment of a full-scale deep biofilm infection. The main hypothesis was that some biomaterials, such as DLC and non-fouling DLC-PDMS-h and DLC-PTFE-h, could be more resistant to bacterial adhesion and biofilm formation than the conventional implant materials. To mimic conditions prevailing in the human body, serum incubations, and dynamic flow conditions are useful in the investigation of bacterial adhesion and biofilm formation *in vitro*.

Under static conditions and in the presence of serum proteins, the DLC was more microbe-repellent than conventional biomaterials tantalum, titanium, and chromium and resisted the most the adhesion of *S.aureus* cells. In this study, it was also found that micropatterned surfaces are useful for quantitative evaluation of bacterial adhesion to biomaterials. Different sizes of microtextures enabled data collection regarding alignment of bacteria on sample surfaces.

Under dynamic flow conditions, DLC and DLC-PTFE-h were as good as conventional biomaterials in their ability to inhibit adhesion of *S.aureus* and *S.epidermidis*. The DLC-PDMS-h bound significantly more *S.aureus* bacteria than DLC-PTFE-h and tantalum. Otherwise, the DLC-PDMS-h behaved similarly to the other materials tested.

Biofilm formation was examined in the presence of serum. The DLC-PTFE-h was superior compared to all materials tested in its ability to resist biofilm formation. The DLC came out next in the ranking order and resisted biofilm formation soundly compared to conventional biomaterials and DLC-PDMS-h. The DLC-PDMS-h was similar to conventional materials in its ability to inhibit biofilm formation.

Antimicrobial peptides (AMPs) are defense proteins of innate immunity found widely in plants, vertebrates, and non-vertebrates with multiple functions even beyond immunity. The cellular sources and expression of AMP human β-defensin-3 (hBD-3) in septic joint implant loosening were examined by immunohistochemistry and double immunofluorescence stainings of peri-implant tissue samples. hBD-3 was associated with monocyte/macrophage-like cells, fibroblast-like cells, and vascular endothelial cells. It was weakly present in foreign body giant cells and not found in neutrophils. The variable topological expression of hBD-3 in peri-implant tissues in septic implant loosening indicated local induction, as has been reported in the literature before.

In summary, the three studies of this thesis suggest that DLC and DLC-PTFE-h coatings could inhibit bacterial adhesion and subsequent biofilm formation, thus preventing infections more efficiently than current implant biomaterials. The cellular sources of the antimicrobial peptide hBD-3 in peri-implant tissues in septic implant loosening are mainly macrophages and vascular endothelial cells.

TIIVISTELMÄ

Lonkkanivelen pitkälle edennyttä nivelrikkoa sekä muita sairaustiloja hoidetaan lonkan tekonivelleikkauksella joka on yksi selkeimmin elämänlaatua parantavista kirurgisista toimenpiteistä. Ikääntyvien ihmisten määrän kasvaessa sekä leikkausindikaatioiden laajentuessa nuorempiin ja aktiivisempiin potilaisiin, implanttien tarve jatkuvasti lisääntyy. Vaikka nykyiset kirurgiset tekniikat sekä leikkaushygienian toteutus ovat pitkälle kehittyneet, tekonivelinfektioita esiintyy 0.5-1.2% ensimmäistä kertaa leikatuista ja uusintaleikkausten jälkeen useammin. Leikkauksen jälkeinen proteesi-infektio on edelleen merkittävä komplikaatio terveydenhuoltojärjestelmässä ja kyseiselle potilaalle luonnollisesti katastrofi johon liittyy huomattavaa kärsimystä, sairastavuutta ja jopa kuolleisuutta. Tekonivelinfektio vaatii usein pitkän antibioottihoidon sekä teknisesti vaativia uusintaleikkauksia parantuakseen. Uusintaleikkauksiin liittyy huomattavia kustannuksia ja pitkiä sairaalahoitojaksoja eikä lopputulos useinkaan vastaa odotuksia. Kasvava antibioottiresistenssi huomioon ottaen ehdotetaan, että optimaalinen tapa ehkäistä näitä infektioita olisi käyttää bakteerien adheesiota sekä biofilmin muodostumista hylkiviä implanttimateriaaleja sekä pinnoitteita.

Timantinkaltaisen hiilen (DLC) ja polymeerien hyödylliset ominaisuudet voidaan yhdistää hiili-polymeeri-hybridipinnoitteissa (diamond-like carbon polymer hybrid eli DLC-p-h). Tähän mennessä on kehitetty kaksi ainutlaatuista timantti-muovi-hybridipinnoitemateriaalia: timantti-polytetrafluoroetyleeni-hybridipinnoite (DLC-PTFE-h) sekä timantti-polydimetyylisiloksaani-hybridipinnoite (DLC-PDMS-h). Edellisessä timantti on siis yhdistetty tefloniin ja jälkimmäisessä silikoniin. Näitä likaa, vettä sekä öljyä hylkiviä hybridipinnoitteita tutkittiin väitöskirjassa.

Tieteellinen Tutkimus Ortonin Timanttiryhmä on valmistanut nämä uudet biomateriaalipinnoitteet itse kehittämällään kaaripurkausmenetelmällä. Kyseisellä menetelmällä valmistetaan myös paksuja sekä korkealaatuisia timantinkaltaisia hiilipinnoitteita (timanttipinnoitteita). Näitä timanttipinnoitteita voidaan käyttää sovelluksissa, joissa tarvitaan korkeaa kuormituskestävyyttä, kuten lonkan tekonivelissä.

Proteesi-infektion ensimmäinen vaihe on bakteerien adheesio eli tarttuminen biomateriaalin pintaan. Tämä tapahtuu lähes välittömästi kun materiaali tuodaan nesteisen bakteeripitoiseen ympäristöön. Bakteeri adheesio johtaa biofilmin muodostumiseen ja mahdollistaa täysimittaisen syvän biofilmi-infektion kehittymisen. Väitöskirjan päähypoteesina oli, että jotkut biomaterialit, kuten DLC sekä DLC-PDMS-h ja DLC-PTFE-h, ovat vastustuskykyisempiä bakteerien adheesiolle ja biofilmin muodostumiselle verrattuna perinteisiin implanteissa käytettäviin biomateriaaleihin. Tutkittaessa bakteerien adheesiota sekä biofilmin muodostumista laboratorio-olosuhteissa (*in vitro*), on hyödyllistä käsitellä materiaalit ensin seerumilla sekä toteuttaa tutkimus virtaavassa nesteessä käyttäen dynaamista virtausmallinnusta. Nämä menetelmät vastaavat paremmin ihmiselimistössä vallitsevia olosuhteita (*in vivo*) jossa kudosnesteet harvoin ovat liikkumattomia ja biomateriaalit pinnoittuvat välittömästi seerumilla ennen ensimmäistä bakteerikontaktia.

Väitöskirjatutkimuksessa todettiin että staattisissa olosuhteissa seerumikäsittelyn jälkeen DLC hylki mikrobeja paremmin kuin tavalliset biomateriaalit tantaali, titaani sekä

kromi ja vastusti parhaiten *Staphylococcus aureus* bakteerin tarttumista biomateriaalipintaan. Tässä työssä nähtiin, että biomateriaalipinnan mikrokuviointi helpottaa bakteerien tarttumisen kvantitatiivista analyysia. Erikokoiset mikrokuviot mahdollistivat myös tiedon keruun liittyen bakteerien sijoittautumiseen näytepinnoilla.

Dynaamisissa virtausolosuhteissa DLC ja DLC-PTFE-h estivät yhtä hyvin kuin tavalliset implanttimateriaalit *S. aureus* ja *Staphyloccous epidermidis* bakteerien tarttumista. DLC-PDMS-h:n pintaan tarttui merkittävästi enemmän *S. aureus* bakteereita kuin DLC-PTFE-h:n tai tantaalin pintaan. Muutoin se käyttäytyi kuten muut testatut materiaalit.

Biofilmin muodostumista tutkittiin seerumin läsnä ollessa. DLC-PTFE-h vastusti näissä kokeissa selvästi parhaiten biofilmin muodostumista. Myös DLC vastusti biofilmin muodostumista hyvin verrattuna tavallisiin implanttimateriaaleihin sekä DLC-PDMS-h:n joiden pintaan biofilmi tarttui herkemmin.

Antimikrobiset peptidit ovat luonnollisen immuniteetin puolustusmolekyylejä, joita esiintyy kasveissa, selkärankaisissa sekä ei-selkärankaisissa eliöissä. Näillä peptideillä on immuniteetin lisäksi muitakin tehtäviä. Ihmisen kudoksissa esiintyy human beta-defensin-3 (hBD-3) nimistä antimikrobista peptidiä. Tässä väitöskirjassa selvitettiin hBD-3:n tuotantoa septiseen nivelimplantin irtoamiseen liittyvissä kudosnäytteissä immunohistokemiallisia menetelmiä sekä kaksoisimmunofluoresenssi-värjäystä käyttäen. Tutkittavat kudosnäytteet oli otettu implanttia ympäröivästä kudoksesta potilailta, joilla oli diagnosoitu septinen nivelproteesin irtoaminen. hBD-3:a löydettiin monosyytti/makrofagi-tyyppisistä soluista, sidekudossoluista sekä verisuonten endoteelisoluista. Sitä oli heikosti nähtävissä vierasesinejättisoluissa eikä lainkaan neutrofiileissä. hBD-3:n vaihteleva topologinen esiintyminen tulehtunutta proteesia ympäröivässä kudoksessa viittaa paikalliseen erityksen, mikä on linjassa aiemman kirjallisuuden kanssa.

Kolme tämän väitöskirjan tutkimusta viittaavat, että DLC sekä DLC-PTFE-h pinnoitteet voivat estää bakteerien tarttumista ja sitä seuraavaa biofilmin muodostumista biomateriaalien pinnalla. Tällä tavoin nämä biomateriaalit ehkäisisivät infektiota tehokkaammin kuin nykyiset implanttimateriaalit. Antimikrobisen peptidin hBD-3:n solulähteet septisessä implantin irtoamisessa ovat pääosin proteesia ympäröivän kudoksen makrofagit sekä verisuonten endoteelisolut.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. The studies are referred to in the text by their Roman numerals (I-IV):

I) Levon J*, Myllymaa K*, Kouri VP, Rautemaa R, Kinnari T, Myllymaa S, Konttinen YT, Lappalainen R. **Patterned macroarray plates in comparison of bacterial adhesion inhibition of tantalum, titanium, and chromium compared with diamond-like carbon**. J Biomed Mater Res A. 2010 Mar 15;92(4):1606-13.doi: 10.1002/jbm.a.32486.

II) Soininen A*, Levon J*, Katsikogianni M, Myllymaa K, Lappalainen R, Konttinen YT, Kinnari TJ, Tiainen VM, Missirlis Y. **In vitro adhesion of staphylococci to diamond-like carbon polymer hybrids under dynamic flow conditions**. J Mater Sci Mater Med. 2011 Mar;22(3):629-36.doi: 10.1007/s10856-011-4231-9.

III) Myllymaa K*, Levon J*, Tiainen VM, Myllymaa S, Soininen A, Korhonen H, Kaivosoja E, Lappalainen R, Konttinen YT. **Formation and retention of staphylococcal biofilms on DLC and its hybrids compared to metals used as biomaterials**. Colloids Surf B Biointerfaces. 2013 Jan 1;101:290-7.doi: 10.1016/j.colsurfb.2012.07.012.

IV) Levón J, Al-Samadi A, Mackiewicz Z, Coer A, Trebse R, Waris E, Konttinen YT. **Human beta-defensin-3 producing cells in septic implant loosening**. J Mater Sci Mater Med. 2015 Feb;26(2):98. doi: 10.1007/s10856-015-5440-4.

*= Equal contribution.

Experimental parts of this thesis were conducted in following places:

Study I) Department of Applied Physics, University of Eastern Finland, Kuopio, Finland; ORTON Research Institute, Diamond Group, Helsinki, Finland; Department of Medicine, Clinicum, University of Helsinki and Department of Internal Medicine and Rehabilitation, Helsinki University Hospital, Helsinki, Finland.

Study II) ORTON Research Institute, Diamond Group, Helsinki, Finland; Laboratory of Biomechanics and Biomedical Engineering, Department of Mechanical Engineering & Aeronautics, University of Patras, Patras, Greece; Department of Applied Physics, University of Eastern Finland, Kuopio, Finland; Department of Medicine, Clinicum, University of Helsinki and Department of Internal Medicine and Rehabilitation, Helsinki University Hospital, Helsinki, Finland.

Study III) Department of Applied Physics, University of Eastern Finland, Kuopio, Finland; Microsensor Laboratory, School of Engineering and Technology, Savonia University of Applied Sciences, Kuopio, Finland; ORTON Research Institute, Diamond Group, Helsinki, Finland; Department of Medicine, Clinicum, University of Helsinki and Department of Internal Medicine and Rehabilitation, Helsinki University Hospital, Helsinki, Finland.

Study IV) Orthopedic Hospital Valdoltra, Valdoltra, Slovenia; Department of Medicine, Clinicum, University of Helsinki and Department of Internal Medicine and Rehabilitation, Helsinki University Hospital, Helsinki, Finland.

ABBREVIATIONS AND SYMBOLS

Agr	accessory gene regulator locus
AMP	antimicrobial peptide
Ar	argon
ASTM	ASTM International, formerly known as the American Society for Testing Materials
ATCC	the American Type Culture Collection
BLAST	basic local alignment search tool
C	carbon
CFU	colony-forming unit
CLSM	confocal laser scanning microscopy
CoC	ceramic-on-ceramic
CoCrMo	cobalt-chromium-molybdenum
CoNS	coagulase-negative staphylococci
Cr	chromium
DAMP	damage-associated molecular pattern
DC	direct-current
DNA	deoxyribonucleic acid
DLC	diamond-like carbon
DLC-PDMS-h	diamond-like carbon polydimethylsiloxane hybrid
DLC-p-h	diamond-like carbon polymer hybrid
DLC-PTFE-h	diamond-like carbon polytetrafluoroethylene hybrid
ECM	extracellular matrix
eDNA	extracellular DNA
EPS	extracellular polymeric substance
FCS	fetal calf serum
Fn	fibronectin
FPAD	filtered pulsed arc discharge method
HAI	healthcare-associated infection
hBDs	1-4 human β-Defensins 1-4
hBD-3	human β-defensin-3
HV	Vickers hardness number
HXLPE	highly cross-linked polyethylene
IAEFM	image-analyzed epifluorescence micros-copy
IDSA	Infectious Diseases Society of America
MoM	metal-on-metal
MoP	metal-on-polyethylene
MRSA	methicillin-resistant *S.aureus*

MRSE	methicillin-resistant *S.epidermidis*
MSCRAMMs	microbial surface components recogni-zing adhesive matrix molecules
MSIS	the Musculoskeletal Infection Society
NaCl	sodium chloride
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	polyethylene
PIA	polysaccharide intercellular adhesin
PJI	prosthetic joint infection
PMMA	poly (methyl methacrylate)
PVD	physical vapor deposition
QS	quorum sensing
SAMs	self-assembled monolayers
S.aureus	*Staphylococcus aureus*
SEM	scanning electron microscope
S.epidermidis	*Staphylococcus epidermidis*
SFE	surface free energy
SS	stainless steel
Ta	tantalum
ta-C	tetrahedral amorphous carbon
TEM	transmission electron microscopy
THA	total hip arthroplasty
THR	total hip replacement
Ti	titanium
TKA	total knee arthroplasty
TSB	tryptic soy broth
UHMWPE	ultra-high molecular weight polyethylene
UV-lithography	ultraviolet-lithography
VRE	vancomycin-resistant *Enterococcus faecium*
VISA	vancomycin insensitive *S.aureus*

R_a	arithmetic mean deviation of the surface
ºC	celsius
θ_C	contact angle
ε	dielectric constant of the electrolyte.
γ_S^D	dispersive component of the surface free energy of the solid
γ_L^D	dispersive component of the surface free energy of the liquid
η	electrolyte viscosity
γ^{lv}	liquid-vapor interfacial energy
γ_S^P	polar component of the surface free energy of the solid

γ_L^P	polar component of the surface free energy of the liquid
γ	shear rate
$\frac{dI}{dp}$	slope of streaming current versus pressure
γ^{sl}	solid-liquid interfacial energy
γ^{sv}	solid-vapor interfacial energy
γ_S	total surface free energy of the solid
γ_L	total surface free energy of the liquid
ε_0	vacuum permittivity
ζ	zeta potential

1. INTRODUCTION

The use of different medical devices and implants in patient care is an important way to support the quality of life and satisfactory functioning health. An implant is a human-made medical device that is used to enhance an existing biological structure, support a damaged biological structure or replace a missing biological structure. Biomedical biomaterials can be applied as a bulk or as coatings on the implants to achieve a proper functionality of the device. There is a countless number of these biomedical applications available today: artificial hip and knee joint implants, artificial heart valves and pacemakers, stents (cardiac catheters), ventilation supporting devices and urinary catheters are used widely in the hospitals. The development of technology in materials science and progress in research have cemented the position of implantology in the field of biomedicine. Ideally, an implant should not cause any undesired reaction from neighboring or distant tissues. Nevertheless, complications can occur during or post-surgery when an invasive device is inserted into the human body. Depending on the type of an implant, these complications may vary and include infection, inflammation, pain, coagulation and the risk of rejection or loosening.

Due to the increasing number of aged people, the development of technology and the widening of the primary operation indications to include younger and more active patients, the use of implants is predicted to grow. Whenever an implant is used in patient care, there is a certain risk of infection, as the implant surface offers a platform for microbial adhesion and biofilm formation. The risk of an infection varies dependently on the type of the implant and location in the human body and is increased with reimplantation (revision). Mortality is highest among patients with cardiovascular implants. In this group, 4-40% of initially inserted implants become infected (Darouiche,RO. 2004). Although the risk of infection associated with primary total hip arthroplasty (THA) is rather low, the total number of THA operations being done is rising markedly. For those 1% of the primary THAs concerned with a prosthetic joint infection (PJI), it is a catastrophic condition associated with patient suffering, significant morbidity, and even mortality (Jamsen,E, et al. 2010). In revision surgery, the incidence of infection is higher, estimated to be 3.2-5.6% in both hips and knees (Montanaro,L, et al. 2011). The enormous economic impact of these infections is seen in increased costs of repeated surgical operations and in both long-lasting hospitalizations and continuing medical care. Each year millions of individuals regain functionality due to orthopedic biomaterials what is reflected also in the global biomaterials market. Orthopedic biomaterials sales were $24 billion in 2007 with an expected growth rate of 7-9% annually (Hallab,N and Jacobs,J. 2013).

Infections begin with bacterial adhesion. Adhesion is related to the survival of microbial species, and the adhesins are among of the most important virulence factors. As infection proceeds, a bacterial biofilm is formed. Bacterial biofilm resembles a complex ecosystem, in which communities of bacterial cells are embedded in structural glycocalyx matrix of polysaccharides, proteins, extracellular DNA (eDNA) and bacterial products (Hall-Stoodley,L, et al. 2004). It has been thought that implant infection, also known as septic loosening, would be less likely if human tissue cells could adhere and grow on biomaterial surface that at the same time could inhibit microbial adhesion (Gristina,AG. 1987).

Currently, strict hygienic protocols are followed and advanced surgical techniques are used. Despite extensive research and progresses in treatment and diagnostics, PJI is still a significant menace both for patient and society. PJI often necessitates a longer course of antibiotical treatment and revision operations. The revision operations require extended hospitalizations and are linked to increased health care costs (Kurtz,SM, et al. 2007). The alarming rise of the antibiotic resistance and the adverse effects associated with the extensive use of antibiotics underline the urgent need for further solutions. Therefore, it is suggested that the optimal way to combat infections of implants would be to use implant materials and coatings able to repel adhesion of bacteria and inhibit biofilm formation. New biomaterials intended for use in medical devices should be tested for their anti-bacterial properties such as their ability to inhibit bacterial adhesion and biofilm formation.

Biomaterial characteristics, bacterial properties, environmental and host factors determine whether initial bacterial adhesion develops into a full-scale implant infection. The diamond-like carbon (DLC) and its novel diamond-like carbon polymer hybrid (DLC-p-h) coatings produced with the Filtered Pulsed Arc Discharge (FPAD) method are promising candidates for utilization in medical implants (Alakoski,E, et al. 2003, Anttila,A, et al. 1999, Lappalainen,R, et al. 2003). Up to date, little information is available in the literature concerning their interactions with bacteria. This thesis aims to analyze an ability of the FPAD-produced DLC and DLC-p-h coatings to repel bacterial adhesion and biofilm formation.

Recent progress in immunology is leading the way for exciting new opportunities in therapeutics and diagnostics (Hogan,S, et al. 2015). Antimicrobial peptides are human body´s natural antibiotics and could offer an advantage in the diagnosis or treatment of implant infections (Batoni,G, et al. 2016, de la Fuente-Nunez,C, et al. 2016, Lee,JK, et al. 2013, Liu,GD, et al. 2014). The cellular sources of antimicrobial peptides in septic joint implant loosening are not well defined yet.

2. REVIEW OF THE LITERATURE

2.1 Implant materials

2.1.1 Overview

An orthopedic implant supports a fractured bone or replaces a part or all damaged, malfunctioning joint. As the demand for better quality of life and number of osteoarthritis patients increases with longer life expectancy amongst older adults, better, durable and long-lasting implants are needed. Particularly in the United States, growing number of obese people and sport accidents enhanced the need for high-quality prostheses (Stoodley,P, et al. 2013). At the same time, the development of materials science and technology allows production of more advanced biomedical devices. Osteoarthritis, rheumatoid arthritis, osteoporosis, fracture and congenital abnormalities are possible indications for arthroplasty. Since use of prostheses can be more than 30 years the current average life span for total hip implants is not sufficient. As the emphasis in this thesis is on orthopedical implant infections, the focus is on biomaterials used in orthopedical devices, mostly joint replacements.

Biomaterials can be classified based on chemical composition (metals, polymers, ceramics, composites and materials of biological origin) and based on materials surface reactivity (inert, with a smooth or a porous surface, chemically reactive surfaces or bioresorbable). The classification of biomaterials can also be done on a historical basis. The first generation biomaterials emphasize a proper functional performance when replacement of body parts is considered, e.g., in load-bearing biomaterials. They have minimal tissue-biomaterial interactions. The second generation biomaterials show control or induction of favorable host reactions, e.g. bioresorbable materials, which allow in-growth of host tissues. The third generation biomaterials is associated with the regeneration of impaired tissue, tissue engineering products and living cells used in combination with artificial materials (Konttinen,YT, et al. 2014).

Orthopedic joint prosthesis materials should maximize wear resistance and fatigue strength and minimize stress shielding and material degeneration due to corrosion or degradation (Kaivosoja,E, et al. 2012a). DeMane proposed properties of an ideal implant: it should be noncarcinogenic, non-allergenic, sterilizable and biocompatible. The material should be clinically inert unless it is biodegradable or fabricated for certain activities. It should also be structurally stable, not physically modifiable by tissue fluids, and not causing inflammatory or foreign body response (DeMane,CQ. 1995).

Biocompatibility is one important property that describes the ability of a material to perform with an appropriate host response (Williams,D. 1987). It means that material has to perform, not only exist in the tissues, the response created by the material has to be appropriate for the application and the nature of the reaction to a particular material, and its suitability may vary from one situation to another (Williams,D. 1999). Both bulk and material surface characteristics contribute to this biocompatibility, and various chemical, biochemical, physiological, physical and other mechanisms affect the outcome (Williams,DF. 2008).

The THA, also named the operation of the century, has been notably successful due to good and predictable long-term results. It started in the 1890s when ivory implants were used in arthroplasties (Gluck,T. 1890). The introduction of metal-on-metal (MoM) THAs, occurred in the 1930s and later they were developed further in the 1950s and 1960s (Wang,W, et al. 2011). The modern low-friction hip arthroplasty era began in the 1970s with the hard-on-soft bearing concept, where polyethylene was used in the cup, stainless steel in the femoral head and stem, and poly (methyl methacrylate) (PMMA) as bone cement (Charnley,J. 1960, Charnley,J. 1979). THA developed by Charnley has been successful ever since, with 77% to 81% survivorship at 25-year follow-up reported in first-generation results of his low friction arthroplasty with the revision of any component as the endpoint. Long-term survival in more than 90% of patients over 60 years of age has been associated with Charnley´s THA (Berry,DJ, et al. 2002, Callaghan,JJ, et al. 2000, Della Valle,CJ, et al. 2004, Wang,W, et al. 2011, Wroblewski,BM, et al. 2007).

Currently, THA usually uses a titanium or cobalt-chromium alloy femoral stem (press fit into place or cemented with PMMA) and a modular cobalt-chrome alloy or ceramic head. The head articulates on a ceramic or ultra-high molecular weight polyethylene (UHMWPE) acetabular cup fitted into a cobalt-chromium or titanium cup liner that is cemented, screwed, or press fitted into place (Hallab,N and Jacobs,J. 2013).

In 2013, there was a total of 22 000 hip and knee arthroplasty operations registered in the Finnish National Institute of Health and Welfare (THL, Terveyden ja Hyvinvoinnin Laitos) Implant Register of which 48% were hip prostheses and 52% knee prostheses. From the year 2000, the number of registered hip-and-knee implants has nearly doubled, especially regarding the number of knee implants (Rainio J,PA. 2014).

2.1.2 Metals

2.1.2.1 General properties of metals

Use of metals in the treatment of fractures started in the nineteenth century with silver wires, iron nails and galvanized steel (Peltier,L. 1990). In the 1940s, the first metallic hip replacement implant was made of CoCrMo alloy (Vitalium) (Plecko,M, et al. 2012). Later on, Co-30Cr-6Mo alloy and stainless steel (SS) became widely used in the main joint replacements and internal fixations.

Metals used as biomaterials in the human body are tightly regulated. Biofunctionality must be clarified using clinical trials. International Organization for Standardization (ISO), American Society for Testing Materials (ASTM) and other standards together with the European Union (EU) /Food and Drug Administration (FDA) guidelines/directives are usually used to verify compliance with “the Essential Requirement” and biocompatibility. Metals remain nowadays highly used in fracture fixations and total joint arthroplasties (TJAs) whenever good load bearing properties are warranted.

Second generation metal- on- metal (MoM) bearings wear rate has been found to be 20 to 100 times lower than that of metal-on-traditional polyethylene (Silva,M, et al. 2005). Large heads in thin acetabular shells reduce incidence of hip dislocation in younger and

more active patients (Manley,MT and Sutton,K. 2008). Even though the MoM couple has biomechanical advantages, the metal ion release has potentially harmful effects. The accumulation of metal ions is disadvantage of using the MoM couples (Dobbs,HS and Minski,MJ. 1980, Ziaee,H, et al. 2007). Increased dermal hypersensitivity to metals and lymphocytic infiltration as a sign of early inflammation with pain has been reported in the surrounding tissues (Dumbleton,JH and Manley,MT. 2005, Willert,HG, et al. 2001, Willert,HG, et al. 2005). There is also a concern of higher cancer risk and occurrence of pseudotumors related to the use of MoM bearings (Willert,HG, et al. 2005). Regarding biological reactivity, metal wear debris is thought to be more reactive than ceramic or polyethylene debris (Dumbleton,JH and Manley,MT. 2005). The use of MoM bearing surface stemmed femoral components has been abandoned in the last years due to high failure rates and concerns explained above (Kumar,N, et al. 2014).

2.1.2.2 Stainless steel-based materials

Stainless steel (SS) materials are used in orthopedics in fracture fixation often temporarily as medullary nails, plates, screws, pins and steel threads. Disadvantages of using SS based materials relate to the release of metal ions and high elastic modulus. Released metal ions may cause various adverse effects like chronic inflammation, carcinogenesis and cell death and necrosis while high elastic modulus causes stress shielding. Wolff´s law states that living bone must be under a certain amount of cyclic stress to remodel and stay healthy. If the stress distribution to bones is abnormal, too high or too low, the bone will remodel into resorption or absorption. This is called stress shielding which can cause weakening of the adjacent bones if the elastic modulus of the material is higher than that of bone.

2.1.2.3 Titanium-based materials

In the 1940s, Ti-based materials started to replace SS and Co-Cr materials in most orthopedic implants. Now Ti and Ti alloys are the most widely used materials in the orthopedic field. In joint implants, pure titanium is sometimes employed in acetabular cups, but usually, $TiAl_6V_4$ or sometimes $TiAl_6Nb_7$ alloys are used for titanium-based acetabular cups and prosthetic stems. As a biocompatible material, titanium has been known for its excellent osseointegration capacity since its early days of use in dental implants. The use of $TiAl_6V_4$ reduces the stress shielding effect and debris generation. This is because the modulus of elasticity of $TiAl_6V_4$ is closer to cortical bone than that of SS or Co-Cr alloys. Ti-based implants can be inserted without bone cement, which is typically used with other metallic and SS and Co-Cr materials to improve stress distribution to the bone. A peri-implant bone around cementless stems has been shown to possess lower risk for osteolysis, and mechanical failure than bone around cemented stems (Affatato,S. 2012). The drawback of titanium alloys relates to their softness compared with Co-Cr-Mo alloys and also to their relatively poor wear and frictional properties. For this reason, titanium alloys are rarely used when hardness or wear resistance is considered to be the optimal property. There has even been a discussion about the link between aluminum (Al) ions released from Ti alloys and Alzheimer's disease, but no conclusive

evidence has been yet provided (Frisardi,V, et al. 2010, Konttinen,YT, et al. 2014, Landsberg,JP, et al. 1992).

2.1.2.4 Chromium-cobalt-based materials

Cobalt-based alloys are used for stems, and as hard metals in the metal head (balls). $CoCr_{28}Mo_6$ (ASTM F75 and F799-11) alloys are most widely used. Co alloys have excellent rigidity, low abrasion and fretting compared to Ti alloys (Revell,P. 2008).Their problems are similar to those associated with SS: the release of metal ions and high elastic modulus.

2.1.2.5 Tantalum-based materials

Tantalum, marketed as Trabecular Metal, is expensive but bone-compatible biometal and has found use in trabecular implants and as an osseointegrating metallic cup shell, which can be utilized as a coating only on $TiAl_6V_4$ or carbon scaffolds. With excellent mechanical properties and bone fixation, tantalum has been shown to be nearly inert (Black,J. 1994). In the case of marked bone loss, tantalum has been used to supplement fixation in total knee arthroplasty (TKA) and THA (Meneghini,RM, et al. 2008, Rose,PS, et al. 2006).

2.1.3 Polymers

2.1.3.1 General properties of polymers

In polymers, which represent the largest class of biomaterials, small molecular size monomers are usually linked to form high molecular weight compounds. Polymers may be derived originally from natural sources or organic synthetic processes.

Most important mechanical properties of orthopedic polymers are yield stress and creep resistance (Hallab,N and Jacobs,J. 2013). They are used commonly as articular bearing surfaces or/and as an interposition cementing material between the implant surface and bone. Synthetic polymers have been widely used for hip and knee implants for decades, PMMA was introduced as a bone cement in 1958, and UHMWPE has been used in implant cups since 1962 (Kaivosoja,E, et al. 2012a). Additionally, polyaromatic polymers such as poly (aryl-ether-ether-ketone) (PEEK) have been recently discussed regarding isoelastic hip stems because of their useful properties like stiffness comparable to the bone (Kaivosoja,E, et al. 2012a).

2.1.3.2 Polyethylene

Polyethylene (PE) is used as tubing for drains and catheters and in orthopedic medicine in ultrahigh molecular weight form as the acetabular component in artificial hips and other prosthetic joints. Beneficial properties of PE include toughness, wear resistance, and

biocompatibility. Radiation sterilization may enhance the strength of PE when done in the inert atmosphere.

In UHMWPE, the increased crystallinity is due to better packing of linear chains, which gives improved mechanical properties. This is beneficial even though both ductility and fracture toughness decrease. The major downside to the use of UHMWPE with metallic heads relates to wear particle generation that in turn causes chronic inflammatory response mediated by macrophages. This response causes the release of lytic enzymes, bone resorbing mediators, and pro-inflammatory cytokines what leads to osteolysis in periprosthetic bone, fixation failure and aseptic loosening of the implant (Maloney,WJ, et al. 1990, Vernon-Roberts,B and Freeman,M. 1977, Willert,HG and Semlitsch,M. 1977).

Cross-linking of polyethylene decreases abrasive, and adhesive wear rates and thermal treatments increase the oxidative stability of UHMWPE (Dorr,LD, et al. 2005, Kaivosoja,E, et al. 2012a). While the data on the clinical performance of highly cross-linked polyethylene (HXLPE) is still limited and controversial, it has gained interest due to its ability to reduce polyethylene wear in TKA and THA (Affatato,S. 2012).

2.1.3.3 Poly (methyl methacrylate) (PMMA)

PMMA was introduced as orthopedic material in 1947 by the Judet brothers, and the first prosthesis was made entirely of PMMA (Judet,J. 1947). These first implants broke frequently, and their use was abandoned.

PMMA is a hydrophobic, hard, rigid and biostable polymer. This amorphous material allows light transmittance and is used nowadays in acrylic bone cement as a filler material or for implant fixation. PMMA has also been utilized as dental filling material and in ophthalmology in intraocular lenses and hard contact lenses (Heath,D and Cooper,S. 2013).

2.1.4 Ceramics

2.1.4.1 General properties of ceramics

The first ceramic material used in THA was alumina (Al_2O_3) in 1970 (Boutin,P. 1972). Nowadays ceramics are used in joint replacements as bearing surfaces or as coatings to improve bone bonding. Ceramics are the most biocompatible materials for bone repair and bone substitute applications due to their excellent inertness. As hard and wear-resistant materials, inert oxide ceramics minimize particle-induced osteolysis (Kumar,N, et al. 2014). Ceramic-on-ceramic (CoC) bearings have low wear rate, are biocompatible and rarely cause allergic reactions compared to metallic prostheses (Wang,A, et al. 2003). Biocompatibility of ceramics comes from ionic bonding and chemical stability. As ceramics are harder than metals and have low wear rates, the CoC bearing couple is a desirable alternative. This bearing couple has a lower coefficient of friction and higher survivorship favoring its use with young and active individuals according to clinical studies (Murphy,SB, et al. 2006).

Nevertheless, reports have been made about weak fracture toughness and low bending strength regarding the use of ceramics in joint replacements (Boutin,P, et al. 1988,

Habermann,B, et al. 2006, Piconi,C and Maccauro,G. 1999). Problems with ceramics are often related to the ceramic bone interface and the insufficient osseous integration of ceramic materials (Hannouche,D, et al. 2005).

With the development of materials science, the wear rates of the new composite ceramic bearings are markedly lower than those of older alumina bearings (D'Antonio,JA and Sutton,K. 2009, Tateiwa,T, et al. 2008). The popularity of ceramic bearings has increased widely due to these lower wear rates and steadily decreasing rates of fracture (nowadays 1 in 2000 over ten years) (Murphy,SB, et al. 2006). Particularly thick DLC coatings made with the FPAD method demand attention regarding orthopedic load-bearing applications. The DLC and the DLC-p-h coatings were investigated in this thesis for their ability to resist bacterial adhesion and biofilm formation.

2.1.4.2 Diamond-like carbon and diamond-like carbon polymer hybrid coatings

Diamond is a crystalline solid carbon (C) allotrope with properties unrivaled by any other known material. Due to its high atom number density and the strong covalent bonding, it is the hardest of materials and the least compressible substance known. In diamond, carbon atoms are bonded with covalent bonds in a certain order. A neutral single C atom has six electrons surrounding the C atom nuclei, out of which four can create chemical bonds with C and other elements. Electron orbitals arrangement in the ground state of C-atom is marked as $1s^2 2s^2 2p^2$ configuration. C can form tetrahedral (sp^3), trigonal (sp^2) and linear (sp^1) bond coordinations (Pierson,H. 1993), where the four ($2s^2$ and $2p^2$) electrons are converted into one out of these three basic hybridization configurations (Figure 1.) (Pierson,H. 1993).

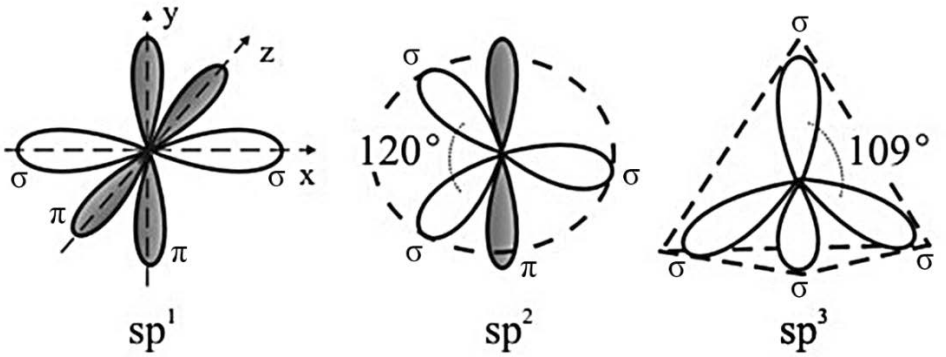


Figure 1. The carbon atom has three hybridization configurations, three possible electron arrangements in the bonding process with C and other atoms. In sp^1-configuration, two out of the four valence electrons enter σ-orbitals, each forming σ-bond directed along the ±x-axis and two other valence electrons form weaker π-bonds (polymers). Sp^2-configuration denotes that carbon forms stable allotrope graphite, where three σ-bonds are in a plane in 120 degrees separation and one π-bond is perpendicular to the plane. In sp^3- configuration diamond is formed, and in this allotrope, four strong

directional σ-bonds with 109-degree angles give the diamond its unique physical properties. Modified from: Soininen A. Studies of diamond-like carbon and diamond-like carbon polymer hybrid coatings deposited with filtered pulsed arc discharge method for biomedical applications. Unigrafia, Helsinki 2015, ISBN: 978-952-9657-78-0.

DLC was produced first time in 1969 by Aisenberg and Chabot (Aisenberg,S and Chabot,R. 1971). It has properties of both graphite and diamond, which is seen in a mixture of sp^2 and sp^3 C bonds and C existing in an amorphous phase. DLC is a general term that describes amorphous carbon coatings with "diamond-like" properties, which have any fraction of diamond sp^3-bonds and varying hydrogen content in the film. Regarding sp^3 diamond bond content in the coatings, the term tetrahedral amorphous carbon (ta-C) refers to coatings where sp^3 diamond bond fraction exceeds 70%. When referred to hydrogenated coatings, hydrogenated ta-C (ta-C: H) can be used. In the case of a low sp^3 fraction, amorphous carbon (a-C) and hydrogenated amorphous carbon (a-C: H) are the preferred terms respectively (Lifshitz,Y. 1999, McKenzie,DR. 1996). In this thesis, the DLC is used to denote only ta-C with a high fraction of sp^3 diamond bonds (>80%) and no hydrogen contained unless otherwise noted. The term DLC polymer hybrid (DLC-p-h) similarly refers to a high fraction of sp^3 diamond bonds in these novel hybrid materials (Anttila,A, et al. 2003).

The Diamond Group at the Orton Research Institute has studied DLC coatings since the mid-80s, and they can produce high-quality, well adherent and extremely thick DLC coatings (sp^3 diamond bonds >80%, thickness >200µm) (Alakoski,E, et al. 2003, Anttila,A, et al. 1999). Superior adhesion of the coatings produced by The Diamond Group is due to the in-house developed Filtered Pulsed Arc Discharge (FPAD) method (Anttila,A. 1989) which is also used by the research group of Professor Reijo Lappalainen at the University of Eastern Finland. Recently, the main focus of the work done by The Diamond Group has been in the investigation of DLC coating for industrial and medical applications. Especially, they have assessed the wear reduction capabilities of DLC coated surfaces in high load-bearing applications such as in THA and hip resurfacing implants (Juvonen T,S, A., et al. Submitted 2015, Lappalainen,R, et al. 2003).

The DLC coatings produced by The Diamond Group and Prof. Lappalainen´s group have been tested in hip simulator studies. In the presence of bovine serum, the wear resistance of hip implants was improved by a factor of 10^6, when they were coated with thick (>10 µm) DLC, and compared to the wear resistance of traditionally used materials (Lappalainen,R, et al. 2003). Additionally, the corrosion of CoCrMo alloy was reduced by a factor of 10^5 in accelerated chemical corrosion studies when it was coated with 1-µm-thick DLC (Anttila,A, et al. 1999, Lappalainen,R, et al. 2003, Tiainen,V. 2001). These results seem promising considering the problems such as the ion release with the MoM prostheses and the high wear rate of the metal-on-polyethylene (MoP) couple.

Commercially available DLC coated cardiovascular implants such as artificial heart valves, blood pumps, and stents exist (Alakoski,E, et al. 2008, Donnet,C and Erdemir,A. 2008, Roy,RK and Lee,KR. 2007), and DLC electrodes are used in biosensors (Freitas,R. 2003). The DLC has been tested successfully as a coating for urinary tract catheters, guidewires, implants in the oral cavity, contact lenses and orthodontic archwires (Donnet,C and Erdemir,A. 2008, Elinson,VM, et al. 1999, Olborska,A, et al. 1994,

Webster,J. 2006). Researchers have also been investigating diamond coatings on drug delivery/sensing devices (Peiner,E, et al. 2007) and bone screws (Koistinen,A, et al. 2005) and work on new future applications of diamond coatings continues vigorously.

In orthopedical load-bearing prosthesis field, so far no commercially successful applications exist. Several articles present DLC coatings as potential alternatives, but at the same time further testing is suggested (Alakoski,E, et al. 2008, Cui,FZ and Li,DJ. 2000, Dearnaley,G and Arps,JH. 2005, Grill,A. 2003, Hauert,R. 2003, Hauert,R, et al. 2013, Love,CA, et al. 2013, Roy,RK and Lee,KR. 2007).

When DLC is used as an implant coating in load-bearing applications, the importance of the adhesion of the films to the substrate comes into question. The coating has to be hard and thick to withstand high mechanical stresses, as in artificial hip and knee joints, when the peak loads are high (more than three times the body weight) (Paul,JP. 1966). Normally, high internal stresses in thick DLC coatings lead to delamination of the coating on hard materials. In the prevention of the delamination and loosening of the films from the substrate, the adhesive forces need to overcome the internal stresses in the films. Delamination of the coating occurs when the internal forces (increased with the thickness and sp^3-fraction of the coating) exceed the adhesive forces. Especially with high-quality ta-C films, the internal compressive stress can be extremely high (Chhowalla,M. 2001, Robertson,J. 1998). Earlier, it has been thought that high-quality ta-C could not be deposited much thicker than 100 nm without increasing the risk of coating delamination from the substrate surface (Anttila,A, et al. 1997, Chhowalla,M. 2001).

The Diamond Group have been able to deposit thick (>200 µm) high-quality DLC films with a proper selection of substrate materials combined with the production of an adequate intermixing layer with high energy carbon ions (Anttila,A, et al. 1999). Essential for the adhesion of the DLC coating to the substrate is the substrate materials ability to form carbides at the material coating interface. It has been shown that the carbide formation in the substrate leads to excellent adhesion of the DLC coatings (Anttila,A, et al. 1995, Anttila,A, et al. 1997, Tiainen,V. 2001, Utsumi,T, et al. 2007). The hardness of the substrate is another essential factor for proper adhesion of the DLC coatings, and the critical hardness limit has been shown to be HV 3 Gpa (Anttila,A, et al. 1997). Below this limit, high-quality DLC coatings at least up to 200 µm in thickness can be produced (Anttila,A, et al. 1999). Above the critical hardness value, the maximum thickness of the highest quality coatings is approximately 500 nm (Alakoski,E, et al. 2003).

The common issues of implant corrosion and wear of the THA could be minimized and delamination prevented with high quality and well adherent DLC coatings. Optimally, DLC could be used on both articulating surfaces, as Alakoski *et al.* suggest in the article which evaluates the use of DLC in load-bearing applications (Alakoski,E, et al. 2008).

Studies done by The Diamond Group have even led to the discovery of an entirely new group of materials, diamond-like carbon polymer hybrids (DLC-p-h). The FPAD system was modified to produce two novel hybrid coatings, DLC polytetrafluoroethylene hybrid (DLC-PTFE-h) and DLC polydimethylsiloxane hybrid (DLC-PDMS-h), where useful properties of DLC and chosen polymers could be combined (Anttila,A, et al. 1999). The Diamond group and the group of Prof. Lappalainen (in collaboration with other

research groups) have published nine articles where these novel coatings have been described and investigated (including articles II and III of this thesis) (Anttila,A, et al. 2003, Calzado-Martin,A, et al. 2010, Huikko,K, et al. 2003, Kinnari,TJ, et al. 2008, Kiuru,M and Alakoski,E. 2004, Lappalainen,R, et al. 2003, Soininen,A, et al. 2014). The novel DLC-p-h coatings are anti-soiling with hydrophobic and oleophobic properties and have a high hardness (Lappalainen,R, et al. 2003). The polymer used and its amount in the resulting DLC hybrid coating determine the final properties of the coating. For hybrids mentioned above, PTFE and PDMS polymers were chosen because they are well known for their non-stick and anti-soiling properties.

2.1.5 Biomaterial surface properties

Implant surface characteristics affect bacterial adhesion and biofilm formation in a complex way. Despite a vast amount of research, much is still unknown how these surface properties influence the outcome of the events. Biomaterial surface factors are interconnected, and many chemical, physiological, physical and biochemical mechanisms are involved. As an example, changing surface chemistry directly also affects the surface charge, zeta-potential and surface energy of the surface. Even the initial protein adsorption is hard to predict due to different affinities and conformational states of the proteins, which material surfaces dictate. Additionally, not only non-specific physical interactions but also specific ligand-receptor mechanisms play a role in bacterial adhesion and biofilm formation.

2.1.5.1 Chemical composition

Biomaterial surface chemistry affects bacterial behavior *in vivo* through protein adsorption. The type of bonds formed between the material surfaces, proteins, and other solutes is regulated via material surface chemical composition, and the material surface dictates both the orientation and conformation of the adsorbed molecules. Therefore, the binding sites available for bacterial and human cellular adhesion are altered through changes in protein structure, which leads to the evolution in human and bacterial cellular binding to the substrate material (Kaivosoja,E, et al. 2012b). Depending on material hydrophobicity and charge, materials with different functional groups alter bacterial adhesion.

2.1.5.2 Surface topography

Surface topography can refer to a surface texture with certain dimensional configurations. Surface topography also describes surface roughness, which is quantified by the vertical deviations of a real surface from its ideal form as a 2-dimensional parameter. Many different roughness parameters exist, and the arithmetic mean deviation of the surface (R_a) is the most common. It does not represent the morphological configurations of the surface. Surface configuration is a morphological description of the pattern of a material surface, and it is a three-dimensional parameter. Monofilament

surface, a braided surface, and a porous surface are examples of particular surface configurations (An,YH and Friedman,RJ. 1998).

Multiple studies have been made in the attempt to explain the relation between bacterial adhesion and surface topography and roughness, but the exact mechanisms remain largely unknown. This is mainly due to limitations in the characterization of substratum roughness and topography properties and complexity of the bacterial surface structures of a given species. Bacterial species, strain type, and bacterial size all influence surface roughness effect.

There have been many reports linking material surface nano-topography with the risk of infection (Crawford,RJ, et al. 2012, Truong,VK, et al. 2015). Despite this, no standard protocols exist for examination of random surface features on implant materials related to bacterial adhesion. Much work has been done regarding the interaction of eukaryotic cells and implant surfaces, but studies evaluating the connection between surface nano-topography and bacterial adhesion are lacking. Crawford RJ has recently proposed a set of topographical parameters for surface roughness characterization in bacterial adhesion studies (Crawford,RJ, et al. 2012).

At the microscale, an optimal feature size may exist due to the limited capability of bacteria to deform. Extracellular structures of the cell membrane like flagella and fimbriae may have a role in bacterial responses to smaller topographical features (Anselme,K, et al. 2010). Finally, one has to consider that changes in surface roughnesses are related to changes in wettability and surface free energy (SFE) properties of a surface which make interpretation of bacterial interaction with surfaces complicated (Das,K, et al. 2009, Khang,D, et al. 2008, Ponsonnet,L, et al. 2003).

Arithmetic mean deviation of the surface (R_a):

$$R_a = \frac{1}{lr}\int_0^{lr} |z(x)|\, dx \qquad (1)$$

Z (x) is the profile from a midline and *lr* is the sampling length over which the surface profile has been measured.

Material surface micro-and nano environment related to bacterial adhesion and biofilm formation is critical in modern implantology. Despite intense research in the area, little is understood of bacterial behavior on surfaces with defined textures. Differences in bacterial species, substrate material, feature aspect-ratio and geometry cause difficulties in interpreting results. The three-dimensional surface topography has been thought to have a significant influence on cell behavior on surfaces (Jager,M, et al. 2007, Martinez,E, et al. 2009). There has been an understanding of specific "contact guidance" related to bone cell alignment along defined substrate morphologies (Charest,JL, et al. 2004, Hamilton,DW, et al. 2006). Recent study reports controlled bacterial mechanic-selective adhesion and inhibition of biofilm growth with the combination of nanoarray-induced bacterial patterning and modulation of the effective stiffness of the nanoarray (Epstein,AK, et al. 2011).

2.1.5.3 Surface energy

Wetting is defined as the ability of a liquid to maintain contact with a solid surface. Cohesive forces cause the drop to avoid contact between the liquid and surface and to ball up, whereas adhesive forces between a liquid and solid enable a liquid drop to spread across the surface. Force balance between these adhesive and cohesive forces determines the wetting property of the surface. The contact angle is an inverse measure of wettability, and it can be defined as the angle that forms when a liquid/vapor interface meets the solid surface. Wettable surfaces are called hydrophilic when water spreads on the surface, whereas non-wettable surfaces are called hydrophobic when a water droplet forms on the surface. Contact angles less than 65 usually describe wettable and hydrophilic properties (high surface energy), and contact angles greater than 65 present hydrophobicity and less tendency to wetting (low surface energy) (Vogler,EA. 1998). The solid surface wettability is influenced by the surface structure and the surface chemistry. Modifications in the surface roughness cause changes in the wettability characteristics of the surface. Patterning increases hydrophobicity in hydrophobic materials and hydrophilicity while in hydrophilic materials.

The surface energy of a homogeneous substance describes the energy required to form a unit area of new surface at the interface. The difference in the tendency of each phase to attract its molecules results in this free energy of the surfaces at an interface (Kaivosoja,E, et al. 2012b). The surface energy of the material is directly related to its wettability properties, which are commonly described by contact angle measurements. Surface energy also depends on chemical composition, crystallographic orientation, and roughness. The interfacial energies (solid-vapor interfacial energy γ^{sv}, solid-liquid interfacial energy γ^{sl}, and the liquid-vapor interfacial energy γ^{lv}) are derived from Young`s equation (Young,T. 1805), where θ is the equilibrium contact angle:

$$\gamma^{sv} = \gamma^{sl} + \gamma^{lv} \cos\theta \qquad (2)$$

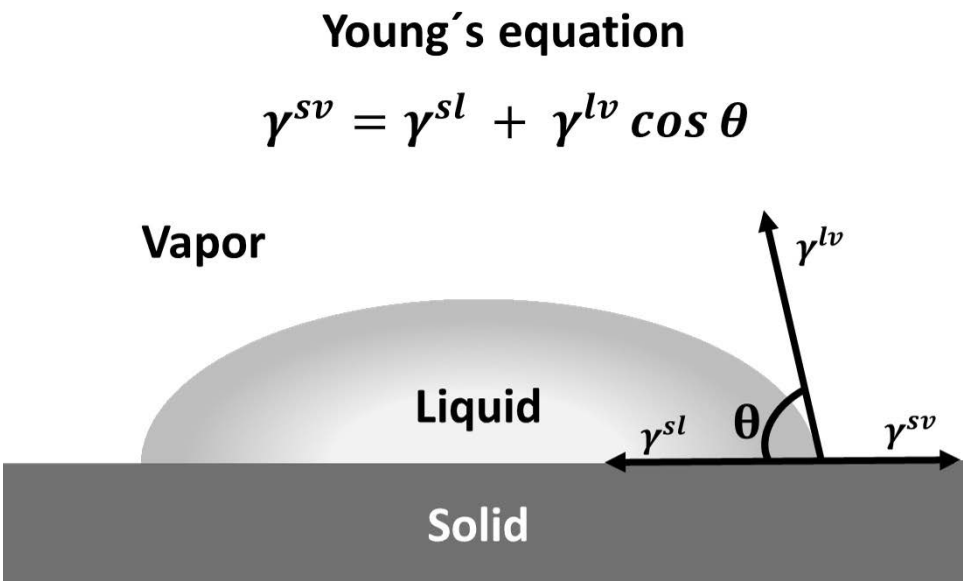


Figure 2. The contact angle and Young's equation used to calculate the interfacial energies**.**

By using different polar and nonpolar liquids whose surface tensions are known in the contact angle measurements, it is possible to calculate a material's surface free energy (SFE) and its components. Hydrogen bonding, dipole-dipole, dipole-induced dipole, and other site-specific interactions are described by the polar component of the material SFE. Van der Waals and other non-site specific interactions between solid surface and a liquid are related to the dispersive component of the material SFE.

2.1.5.4 Zeta potential

The zeta potential (ζ-potential) describes the potential difference across phase boundaries between solids and liquids. An electric double-layer is formed when a solid surface is in contact with a fluid and surface charges accumulate. The zeta potential, or electrokinetic potential, is used to quantitate the magnitude of the electrical charge at the double layer. Near the solid-liquid interface, the charge carriers are fixed in the stationary layer, whereas, in the liquid phase, the charge carriers are at greater distances and they are mobile (mobile layer). These layers are separated by a plane of shear and the potential at this interface between immobile and mobile layers is referred to as the zeta potential. It is a measure of charges attracted by the material surface suspended in a liquid.

Zeta potential is one of the properties that may have a significant role in bacterial adhesion/biofilm formation on the surface. The presence of polyelectrolytes such as proteins and glycosaminoglycans in the dispersion fluid influence the zeta potential.

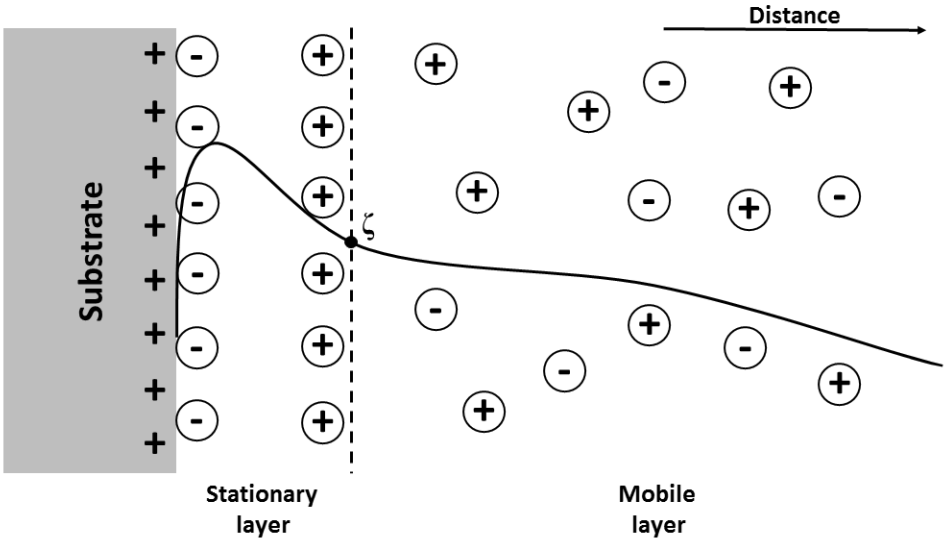


Figure 3. A schematic presentation of the electrostatic double layer and the charge distribution. The zeta potential refers to the potential decay at the shear plane, between stationary and mobile layers.

2.1.6 Debris generation and ion release

Regarding implant performance in clinical use, implant material degradation and debris formation by wear and corrosion and host response to this debris are important. Wear describes the loss of material in particulate form as a consequence of relative motion between two surfaces. With corrosion, material gradually destructs by chemical reaction with the environment. This way metal is converted to its more stable oxide. The degradation products (debris) of all orthopedic implants can be wear particles, colloidal nanometer size complexes bound by protein, soluble debris (metal ions), and inorganic metal salts/oxides or in an organic storage form such as hemosiderin (Hallab,N and Jacobs,J. 2013).

Primary local effect of implant debris by wear and corrosion is osteolysis around the implant. The exact mechanism by how sterile, relatively inert implant debris causes inflammation is unclear, and considerable efforts are on the way in research. When remote and systemic effects of debris are considered, many unsolved issues remain. While wear and electrochemical corrosion degrade useful properties of materials, the resulting debris has been associated with adverse effects in human body. Remote and local adverse effects and host response to debris formed by wear or electrochemical corrosion might be determined by host factors and the amount, quality, and type of debris created (chemical form). Additionally, different orthopedic materials and couples produce different kinds and quantities of debris (Hallab,N and Jacobs,J. 2013).

Particulate wear debris accumulation in the tissues is followed by inflammatory reaction and local bone loss (aseptic osteolysis) which can eventually lead to implant failure. This particulate debris may also be phagocytosed within macrophages and migrate

via lymphatics to distant organs and lymph nodes. It may cause granulomatous lesions, lymphadenopathy, inflammation, and fibrosis. With wear rates of more than 0.1 mm/year, osteolysis occurs more commonly, and it is uncommon with wear rate less than 0.05 mm/year (Dumbleton,JH, et al. 2002). MoP couples generally produce more wear than hard-on-hard material couples such as metal-on-metal articulations. The macrophage reactions to debris have been seen to determine debris-induced inflammation that is the most leading cause of implant loosening over time (Hallab,N and Jacobs,J. 2013). In long-term use, aseptic osteolysis accounts for over 75% of implant failures in TJA (Holt,G, et al. 2007).

During implantation or wear or upon chemical interaction with a biologic environment, metal ions can be released through corrosion. On the infected substratum, iron may accumulate and supply bacteria and inhibit macrophage function. Iron has been associated with virulence of *S.aureus* and *S.epidermidis* (Gristina,AG. 1987). Trace ions (e.g. Mg 2+, Ca 2+) in microzones present on implant surface may enhance cell-to-cell and cell-to-surface binding in biofilms and increase resistance to external threats (Dankert,J, et al. 1986).

Metal ions released from the implant may bind to specific proteins and disseminate via lymphatics or circulation and accumulate in organs such as spleen or liver. There have been reports about the elevated serum and urine levels of some metal ions after THA operation. Implant alloys in modern orthopedics include elements potentially toxic to the human body: titanium, aluminum, vanadium, cobalt, chromium and nickel. In soluble form and excessive amounts, adverse effects may follow. There have also been reports of immunologic type responses temporally associated with implanted metal components. Metal hypersensitivity is common and has been linked to ingestion or dermal contacts with metals. Dermal hypersensitivity manifests as skin hives, eczema, redness, and itching.

Metal toxicity of debris may have several patterns: it may alter metabolic events, change host/parasite interactions, act as haptens and activate immunological responses and be anti-chemotactic and finally cause chemical carcinogenesis. Nevertheless, at this time any metal release related to metabolic, bacteriologic, immunologic or carcinogenic toxicity is still without confirmation because cause and effect relation has not been well-established in human subjects (Hallab,N and Jacobs,J. 2013).

2.2 Protein adsorption and conditioning film

In an aqueous environment, as in human body, molecules, inorganic or organic are carried to the surface either by diffusion or turbulent flow (Palmer,J, et al. 2007). The accumulation of molecules at the solid-liquid interface on surfaces is called a conditioning film. Whenever an implant is inserted into the human body, and it comes into contact with tissue fluids or blood; first the surfaces are covered by water molecules (in nanoseconds) (Roach,P, et al. 2007). The organization of water molecules at the material-liquid interface depends on physicochemical properties of the surface. Water molecules are more organized at hydrophilic surfaces, which have strong tendency to bind water. On hydrophobic surfaces water molecules are less organized concerning hydrogen bonding;

thus, hydrophobic surfaces have a weak tendency to bind water. After the formation of the hydration shell, an adsorbed water layer, hydrated ions get involved according to properties of the surface. Glycoproteinaceous conditioning film is formed as the implant surface is immediately (in seconds) coated with plasma proteins, platelets and various extracellular matrix molecules such as fibronectin, collagen, and fibrinogen derived from tissue fluids, blood, and ECM. Bacterial and tissue cell adhesion is subsequently possible via this conditioning film (Dankert,J, et al. 1986).

In vivo, the conditioning film forms before microorganisms or tissue cells adhere because adsorption of microbes and tissue cells to a substratum proceed relatively slow compared to that of molecules (Escher,A and Characklis,W. 1990, Gristina,AG. 1987). The affinities of the proteins and surfaces, as well as the mass concentration of the proteins in the bulk phase, decide the outcome of the competitive adsorption of proteins to the surface. This explains why the concentration of low-affinity protein albumin, adsorbed from plasma on the surface, is about the same as that for fibrinogen. The concentration of albumin in plasma is much higher than that of high-affinity fibrinogen. Physicochemical characteristics of the surface, especially wettability (hydrophilicity or hydrophobicity), play a role in this process (Wilson,CJ, et al. 2005). Proteins might also change their conformation (folding of proteins) when adhering to the surface and this way their biological functions may be altered. This relates to protein stability, which affects protein adsorption. So-called soft proteins have a low internal stability and unfold more smoothly during initial adhesion. Hard proteins have high internal stability and unfold less easily. Thermodynamically labile proteins are more adsorptive than stable proteins due to their unfolding in response to denaturing conditions more easily. Generally, on hydrophobic surfaces, protein adsorption is greater compared to adsorption on hydrophilic surfaces (Wilson,CJ, et al. 2005). When adhering to the hydrophilic surface, proteins retain their natural conformation better.

The physicochemical properties of the surface (surface free energy, hydrophobicity, and electrostatic charges) are altered then by the conditioning film. The protein layer has a major role both in bacterial and tissue adhesion (Dickson,JS and Koohmaraie,M. 1989) because the adhering bacteria and tissue cells come into contact first with a conditioning film and other cells and their products rather than a bare substratum surface.

2.3 Implant infections

2.3.1 Overview

Surgical operations are always associated with a particular risk of complications. With arthroplasty, possible complications and reasons for revision surgery due to the failure of the primary implant include infection, poor biocompatibility, dislocation, instability, fracture, poor surgical technique and wear/corrosion. Often the implant failure is related to the mechanical properties of the implant. Such conditions include wear/corrosion, low fracture toughness/low fatigue strength, and stress shielding, where there is a mismatch in elastic modulus of implant material compared to that of bone (Geetha,M, et al. 2009). According to Wolff´s law (Wolff,J. 1892), when rigidly fixed stiff femoral components

shield the bone from stress, the resorption of proximal femoral bone may increase in lack of loading that would strengthen the bone.

Wear and corrosion induced particle debris can predispose the periprosthetic tissues to chronic inflammation and foreign body reaction, which ultimately may lead to osteolysis and pathologic fracture. Other cause of aseptic loosening of the implant can be poor integration between implant surface with bone and surrounding tissues due to micromotions in the case of inefficient primary stability of the implant, which may lead to fibrous encapsulation.

Prosthetic joint infections (PJIs) occur more rarely than aseptic failures, but the consequences are much more dramatic due to the high morbidity and health-care costs associated with the complication (Waldwogel,F and Bisno,A. 2000). Due to better detection methods in diagnostics, increasing number of implanted prostheses amongst seniors and younger people involved in sports accidents, the incidence of PJIs is expected to rise. Additionally, with longer expected residency time of prostheses, infections become even more widespread.

The risk of an implant infection is dependent on the type of the implant and location in the human body and is increased with reimplantation (revision). In Mayo Clinic survey between 1969 and 1996, the prevalence of PJIs for primary THA or TKA was 1.3% or 2.0% respectively. For revision THA or TKA, the prevalence was 3.2% or 5.6% (Hanssen,A and Rand,J. 1999). Among orthopedic devices the infection rate with primary hip replacement patients during the first two years after initial implantation is usually less than 1% and in those with knee replacement less than 2%. After revision surgery, the infection rates are much higher (5-40%) than after primary replacement (Trampuz,A and Widmer,AF. 2006). In the Nordic Arthroplasty Register Association (NARA) database, 432 168 primary THAs were registered between 1995 and 2009 of which 2778 (0.6%) were revised due to infection (Dale,H, et al. 2012). In 2013, of 1777 registered THA revisions in Finland, 10.8% were performed because of an infection (Rainio J,PA. 2014). About 45% of all nosocomial infections are implant-related (Schierholz,JM and Beuth,J. 2001) and the annual costs for approximately 2 million nosocomial infections were near $11 billion in the United States in 2001 (Schierholz,JM and Beuth,J. 2001).

The focus in pathogenic agents in this thesis is mainly on two staphylococcal species, *S.aureus,* and *S.epidermidis,* which are most often associated microorganisms with orthopedic implant infections. Approximately two-thirds of orthopedic implant infections are caused by either *S.aureus* or *S.epidermidis* (Campoccia,D, et al. 2006). Among the PJIs, 20-40% is hematogenous, and the rest are from direct contaminations. Most often PJIs are derived from contamination of the surgical wound (Dellinger,E, et al. 2007)

The detailed clinical diagnostic and treatment processes of PJIs are out of the scope of this thesis. However, for successful treatment of implant infections, it is often necessary to remove the implant and administer antibiotics for a longer period before reimplantation to avoid recurrent infections at the same site. Even low bacterial counts can contaminate the implant or surgical wound, due to the presence of abiotic foreign bodies and impaired local host responses. Therefore, it is impossible to insert the implant under 100% sterile conditions even with modern operating rooms, where strict procedures are followed by

experienced operating teams. This means that contamination of an implant might be almost unavoidable, and it is indeed the consensus today that the incidence of microbial contamination of the implants is considerably underestimated.

As active players in the host defense settings, antimicrobial peptides (AMPs) are human body´s natural antibiotics. Their presence and production have been associated with several inflammatory and infectious processes, including septic joint implant loosening. In recent years, their functions have been reported to link innate and adaptive immunity. Of particular interest is human β-defensin-3 (hBD-3), which belongs to the largest AMP family of defensins. In literature, hBD-3 has been studied regarding its antimicrobial activity and possible utilization in diagnostics (Lee,JK, et al. 2013, Liu,GD, et al. 2014).

2.3.2. Definition

Deep joint implant-related infections are difficult to recognize especially when pain is the only notable symptom. The Musculoskeletal Infection Society (MSIS) has agreed on criteria (published in the November 2011 issue of the Journal of Clinical Orthopedics and Related Research (Parvizi,J, et al. 2011), which should be met for definite PJI diagnosis. The International Consensus Meeting (ICM) on PJI supported the MSIS definition of PJI in August 2013 and modified it by adding a minor criterion (leukocyte esterase test) and determining the threshold for lab results, Table 1, (Parvizi,J, et al. 2013, Parvizi,J, et al. 2014, Shahi,A and Parvizi,J. 2015).

The modified definition states that a definite PJI is present when following criteria are met:

Table 1. The definition of a prosthetic joint infection.(Parvizi,J, et al. 2014).

A. There is a sinus tract communicating with the prosthesis, OR;
B. A phenotypically identical pathogen is isolated by culture from 2 or more separate tissue or fluid samples obtained from the affected prosthetic joint, OR;
C. When three of the following five criteria are present:
    1. Elevated serum erythrocyte sedimentation rate AND serum C-reactive protein concentration
    2. Elevated synovial white blood cell count, OR ++ change on leukocyte esterase test strip
    3. Elevated synovial polymorphonuclear percentage
    4. Positive histological analysis of periprosthetic tissue
    5. A single positive culture

The definition is based on the demonstration of a causative microbe associated with clear signs of the host inflammatory response. In some low-grade infections, several of these criteria may not be routinely met despite the presence of PJI.

Another large international workgroup, the Infectious Diseases Society of America (IDSA), recently published also a consensus document regarding the standardization of the definition of PJI (Osmon,DR, et al. 2013). Considering the history and physical examination, according to IDSA, PJI should be suspected in patients with certain conditions. These conditions include a sinus tract or persistent wound drainage over a joint; acute onset of a painful prosthesis; or a chronically painful prosthesis at any time postoperatively. Particularly a chronically painful prosthesis postoperatively is alarming in the absence of a pain-free period in the first few years after implantation. Also, if a patient presents with a chronically painful prosthesis postoperatively with a history of wound-healing problems or a superficial wound infection, further evaluations are ordered (Osmon,DR, et al. 2013).

2.3.3 The staphylococci

2.3.3.1 Overview

Immobile, gram-positive staphylococci are 0.5-1.5 μm in diameter and are characterized by individual cocci forming grape-like clusters in more than one plane. Pathogenic staphylococci such as *S.aureus* can clot blood and produce coagulase (Kloos,WE and Musselwhite,MS. 1975) and are in this way often identified distinguishing them from coagulase-negative staphylococci (CoNS) like *S.epidermidis, S. lugdunensis,* and *S.Saprophyticus.*

The staphylococci are often found in the normal flora of the human skin and mucous membranes. Staphylococcal cell wall consists mainly of peptidoglycan and phosphate-containing polymers called teichoic acids. Through localization of metal ions and the activities of autolytic enzymes, a negative charge is created on the staphylococcal cell surface by these teichoic acids (Gross,M, et al. 2001). Additionally, the cell wall includes surface proteins called adhesins, exoproteins (exotoxins) and peptidoglycan hydrolases (autolysins). In *S.aureus* and *S.epidermidis*, teichoic acids and the surface protein autolysin have been associated with abiotic surface attachment (Gross,M, et al. 2001, Heilmann,C, et al. 1997). They probably act indirectly, through alteration of surface hydrophobicity (Joo,HS and Otto,M. 2012).

Bacterial capsules and slime are both subclasses of extracellular polymeric substances (EPS) and are usually polysaccharides (An,YH and Friedman,RJ. 1998), which can bind to surfaces or surface adsorbates and may function in cell-to-cell aggregation. Bacterial capsules are separated from extracellular slime as a discrete covering layer with clear margin on the bacterial cells outside of the cell wall, and they can be found both in Gram-negative and Gram-positive bacteria. Bacterial strains that do not produce slime have been shown to be less pathogenic (Davenport,DS, et al. 1986).

S.aureus and *S.epidermidis* adhere to cells, plasma and extracellular matrix (ECM) proteins such as fibronectin, fibrinogen, collagen, vitronectin and laminin (Foster,TJ and McDevitt,D. 1994) with microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)- adhesins (Patti,JM, et al. 1994). This is significant especially

in wounds and foreign body infections. One single MSCRAMM can bind several ECM ligands while a microorganism can express several MSCRAMMs that bind into the same matrix molecule (McDevitt,D, et al. 1994). Some MSCRAMMs have been identified, such as fibronectin binding proteins, clumping factor binding proteins and collagen binding proteins (Herrmann,M, et al. 1988). It has been reported that polysaccharide intercellular adhesin (PIA) maintains connections between bacteria in biofilm and plays a vital role in biofilm formation (Mack,D, et al. 1996b).

S.aureus and *S.epidermidis* cause most of the implant infections. Especially CoNS such as *S.epidermidis,* which mainly is associated with foreign body infections, are implicated. New causative agents resistant to antibiotics have evolved such as MRSA and vancomycin insensitive *S.aures* (VISA). Also, *S.epidermidis* has shown high resistance to antibiotics and nosocomial infections caused by *S.epidermidis* are often very hard to treat.

The survival and growth of staphylococci are possible only with adaptation to environment. This adjustment is maintained by multiple mechanisms including bacterial adhesion, phenotype change, biofilm formation, changes in metabolic state of the bacteria, quorum sensing (communication and signaling between bacteria) and various virulence factors. The virulence factors of *S. aureus* and *S.epidermidis* include structures of the microbial cell, secreted products by the microbe and the genetics of antibiotic resistance.

2.3.3.2 *Staphylococcus aureus*

S.aureus often causes metal-biomaterial, bone-joint and soft-tissue infections (Barth,E, et al. 1989, Petty,W, et al. 1985). It is a virulent pathogen among immunocompromised and healthy individuals because it produces extracellular toxins (enterotoxin A-E, toxic shock syndrome toxin-1 (TSST-1)), extracellular proteases (metalloproteases, serine proteases V8 (SspA)), and exfoliative toxins (Projan,S and Novick,R. 1997).

S.aureus is present on the skin and in the nasopharynx of the human body and is the pathogen in many minor local infections of the skin, nose, urethra, vagina and gastrointestinal tract (Shulman,J and Nahmias,A. 1972). Surgical wound infections can be caused by *S.aureus* as well as more severe disease states such as endocarditis, osteomyelitis, toxic shock syndrome and even septicemia through lymphatic channels or blood. *S.aureus* causes characteristic local abscess lesions in the tissues underlying the skin (Elek,SD. 1956).

Through plasmids and genetic spread resistance to antibiotics MRSA has evolved and is nowadays a common problem in the hospitals around the world, even though in Nordic countries it is still quite a rare pathogen. MRSA strains produce penicillin binding protein (PBP2) with low affinity to β-lactam antibiotics in such a way that the cell wall of bacteria can be synthesized even in the presence of the antibiotic (Hiramatsu,K. 1995). Vancomycin glycopeptide has been the antibiotic drug of choice against MRSA infections. Nevertheless, since 1996 several VISA strains have been reported (Spagnolo,AM, et al. 2014).

2.3.3.3 *Staphylococcus epidermidis*

S.epidermidis is the most important CoNS species, covering 65-95% of all staphylococci in the skin and mucous membrane normal flora and is often causative agent in polymer-associated implant infections (von Eiff,C, et al. 2002). It is widely distributed in nose mucosa, perianal region, loins, armpits and between toes. *S.epidermidis* is one of the most often isolated microorganisms in hospitals and covers most implant infections caused by CoNS. It has been found to be more resistant to antibiotics than *S.aureus* (Johnson,AP, et al. 2003, Vuong,C and Otto,M. 2002). The rise of methicillin-resistant *S.epidermidis* (MRSE) (Tammelin,A, et al. 2000) and intermediate vancomycin resistance (Raad,I, et al. 1998) create difficulties in the eradication of these infections.

As *S.epidermidis* and other CoNS excluding *Staphylococcus saprophyticus* are opportunistic bacteria, they do not cause infections in normal circumstances. Otto M termed *S.epidermidis* as "accidental pathogen" pointing out its nature as opportunistic microbe (Otto,M. 2009). Many virulence determinants of *S.epidermidis* are thought to have original functions in its commensal non-infectious lifestyle on the skin and the normal flora. Exposure to an immunocompromising agent such as a skin wound, implant or chronic disease is needed in case of "accidental" infection caused by this opportunistic microorganism. The frequency of possible contamination events and mechanisms affect more to the clinical importance of *S.epidermidis* infections than its non-infectious lifestyle. *S.epidermidis* possesses more determinants promoting persistence (such as immune evasion molecules), rather than those associated with aggressive anti-host attacks such as toxins (Otto,M. 2009), which clarifies the low virulence of *S.epidermidis*. *S.epidermidis* is less cytotoxic and virulent than *S.aureus* even though it produces extracellular toxins such as δ-toxin and proteases (von Eiff,C, et al. 2002).

When *S.epidermidis* adheres to an implant surface, it starts to form a biofilm, which is characteristic for *S.epidermidis*. In the biofilm, bacterial cells are attached to each other with PIAs, which are essential for biofilm formation and host attack evasion (Mack,D, et al. 1996b, Vuong,C, et al. 2004b).

2.3.4 Bacterial adhesion

2.3.4.1 Definition and mechanisms

Bacterial adhesion is defined as a process where bacteria adhere firmly to a biotic or abiotic surface by complete physicochemical interactions between them. Initial phase of reversible physical contact is followed by time-dependent phase of irreversible chemical and cellular adherence (An,YH and Friedman,RJ. 1998). Co-aggregation is a term that describes adhesion of planktonic bacteria to each other and co-adhesion means adhesion of planktonic single or co-aggregated microbes to surface-bound sessile microorganisms, sometimes called primary colonizers (Whittaker,CJ, et al. 1996). The sessile surface-bound bacteria can stimulate adhesion of other, still suspended planktonic microbial cells. This can happen either by slowing them down while they are approaching the surface

(Busscher,HJ, et al. 1991, Dabros,T. 1989) or through strong attractive interactions as in co-adhesion (Bos,R, et al. 1995).

As biological systems are diverse and bacterial adhesion is a highly complicated and multifactorial process, it is not completely understood even though various theories have been made to explain the phenomenon. The outcome of the process seems to be determined by physical and chemical interactions that are attractive or repulsive, depending on the interplay among the chemistries of the bacterial and substratum surfaces and the aqueous phase. Bacterial properties, environmental properties, and surface properties all contribute to the process. Cell surface proteins, polysaccharides, conditioning films on surfaces, and biological changes in adhering bacteria seem to affect bacterial adhesion in a way that prediction of the process is virtually impossible based only on the physicochemical models of the different theories (Katsikogianni,M and Missirlis,YF. 2004). Hence experiments *in vitro* and *in vivo* are needed.

2.3.4.2 Phase one: initial adhesion

The reversible and initial non-specific phase of bacterial adhesion involves physicochemical forces. First, planktonic bacteria move to or are driven to a material surface by the effects of physical forces such as van der Waals attraction forces, gravitational forces (sedimentation), Brownian motion, the effect of electrostatic surface charge, hydrophobic interactions or a convective mass transport (Gottenbos,B, et al. 2002).

Concentration gradients of diffusible ("chemotaxis") or surface bound ("haptotaxis") chemical factors, chemoattractants (e.g. amino acids, sugars, oligopeptides) can direct movement of bacteria (diffusion), and specially chemotaxis can modulate bacterial growth on surfaces. Chemotaxis regulates cellular adhesion components and prepares cells for cell-cell and cell-surface interactions (Jenal,U. 2004). The intrinsic motility of a microbe has a role in the microbial movement as they can even actively move with surface-associated appendages such as flagella.

The physical interactions have been further classified as long-range interactions and short-range interactions (Gottenbos,B, et al. 2002, Katsikogianni,M and Missirlis,YF. 2004). Bacteria are transported to the surface with nonspecific long-range interactions (distances > 50nm) which are a function of the distance and free energy (van der Waals forces, electrostatic interactions). Short-range interactions such as ionic and dipole interactions, chemical bonds (e.g. hydrogen bonds), and hydrophobic interactions become effective with closer contact to the surface (< 5 nm) (Mayer,C, et al. 1999), where specific adhesion receptors may facilitate strong adhesion.

The initial early phase can be described as a reversible attachment while the late phase involves firmer adhesion with the surface through molecular reactions between bacterial surface structures and substratum surface.

2.3.4.3 Phase two: late adhesion

Specific irreversible adhesion can be defined as the selective binding between bacterial adhesin (a specific molecular component on a bacterial surface) and substratum receptor

(a specific component on a material surface or tissue surface). This late adhesion is less affected by many common environmental factors such as electrolytes, pH, or temperature (An,Y, et al. 2010).

Specific adhesion of bacteria is believed to be important in the pathogenesis of prosthetic infections (Timmerman,CP, et al. 1991). Bacterial surface polymeric structures such as capsules, fimbriae or pili, and slime mediate this specific irreversible phase of bacterial adhesion (Dunne,WM,Jr. 2002). Their functional parts, adhesins, make specific and secure connections with the surface (especially when the substratum are host tissues) in this time-dependent phase that involves molecular and cellular interactions (Gotz,F. 2002, Mack,D. 1999, O'Gara,JP and Humphreys,H. 2001). Bacterial fimbriae have been associated with virulence (Hacker,J. 1992). In addition to specific binding, they may also react nonspecifically with inorganic substratum elements by charged or hydrophobic interactions (Gristina,AG. 1987).

MSCRAMMs are well-studied adhesins of staphylococci and some other bacteria. They play a major role in this irreversible adhesion and subsequent formation of multi-layered bacterial biofilm. Along with intercellular adhesion, this especially involves binding to host tissues and conditioning film.

2.3.4.4 Bacterial properties

Different bacterial strains and species adhere differently to substratum since they have different physicochemical characteristics. Physicochemical characteristics of bacteria depend on components and molecules (e.g. adhesins, different proteins, polysaccharides and ionic groups) of bacterial surface structures such as fimbriae, teichoic acids, capsule and cell wall. These characteristics are also affected by environment (e.g. growth medium, the ionic strength of the medium, other microorganisms, and surface material properties).

Hydrophobicity has been thought to be a major factor in bacterial adhesion and refers to the organization of water molecules in the region near any surface in aqueous solution. Bacteria with hydrophobic properties prefer hydrophobic material surfaces, and the ones with hydrophilic characteristics prefer hydrophilic surfaces (Katsikogianni,M and Missirlis,YF. 2004), and hydrophobic bacteria adhere to a greater extent than hydrophilic bacteria (van Loosdrecht,MC, et al. 1987).

Bacteria in aqueous suspension are always negatively charged due to the ionization of their surface groups (Hogt,AH, et al. 1985), and they have a net negative zeta-potential on their cell wall at neutral pH (Gilbert,P, et al. 1991). Growth medium, the pH and the ionic strength of the suspending buffer, bacterial age, and bacterial surface structure influence the surface charge of the bacteria which varies according to bacterial species (Dankert,J, et al. 1986). The charge on the bacterial cell surface often referred to as its zeta-potential or electrokinetic potential, is calculated from the mobility of the bacterial cell in the presence of an electrical field under defined salt concentration and pH.

In the case of multiple bacterial species or strains in the tissues or on the substratum, the severity of infections is increased (Merritt,K. 1988). The presence of pre-adhered

organisms has been shown to enhance biofilm mass and bacterial adherence *in vivo* and *in vitro* (Chang,CC and Merritt,K. 1991, Chang,CC and Merritt,K. 1994).

2.3.4.5 Biomaterial properties

Material surface characteristics such as chemical composition, surface charge, hydrophobicity, surface roughness and physical configuration influence bacterial adherence to a biomaterial surface. Biofilms and conditioning film alter material surface energy, empty binding sites, and hydrophobicity (Gristina,AG. 1987). It has been seen that in adhesion *S.epidermidis* prefers polymers and *S.aureus* metals (Gristina,A, et al. 1987).

Material surface chemistry has an effect on bacterial adhesion due to different functional groups of materials which change material hydrophobicity and charge. This has been shown in work done by Tegouila and Cooper (Tegoulia,VA and Cooper,SL. 2002). *S.aureus* adhesion on self-assembled monolayers (SAMs) terminated with methyl, hydroxylic, carboxylic acid and tri (ethylene oxide) was investigated. The adhesion was lowest on ethylene oxide-bearing surfaces (EG3), followed by the hydroxyl surfaces, and higher on carboxylic-and methyl-terminated SAMs. Bacterial adhesion can be inhibited with surface chemistry modifications. Silver (Silver,S. 2003), DLC (Hauert,R. 2003) and plasma coatings (Whitehead,KA, et al. 2004) have been used to decrease the number of adherent bacteria on material surfaces. Peptide coatings (Harris,LG, et al. 2004) and nonsteroidal anti-inflammatory drug coatings (Baveja,JK, et al. 2004) also have been shown to hinder bacterial adhesion.

Surface roughness is commonly described as the arithmetic average roughness. Many groups have reported greater cell attachment on surfaces with high surface roughness and irregularities on surfaces, due maybe to increased surface area and the depressions in the roughened surfaces, which provide more sites for colonization (Baker,AS and Greenham,LW. 1988) and also protect from cleaning chemicals and fluid forces. Therefore, smoothening of the surface can reduce biofilm formation (Ionescu,A, et al. 2012). A roughness level R_a equal to 0.2 μm was suggested to be the threshold value for maximum reduction of bacterial adhesion (Quirynen,M, et al. 1996). Nevertheless, a universally optimum roughness value has not been found that could reduce adhesion of all bacterial species (Renner,LD and Weibel,DB. 2011). The specific effects of surface roughness on bacterial adhesion and biofilm formation vary with the size and shape of bacterial cells and other environmental factors.

Physical surface configuration is a morphological description of the pattern of a material surface, such as a monofilament surface, a porous surface, or gridlike surface, and it is a 3-dimensional parameter routinely evaluated by scanning electron microscopy (An,YH and Friedman,RJ. 1998). Bacteria adhere more to porous, grooved and braided materials compared to flat ones, probably partially due to increased surface area (Bos,R, et al. 2000). Surface topography and irregularities around the critical size close to the diameter of the microbes may entrap bacteria and provide protection from fluid flow and increase the retention forces of adherent bacteria and biofilm on the surface. Grooves or scratches that are close to the bacterial size increase the contact area and binding, whereas grooves that are much larger than the bacterial size show binding potential of a flat surface.

Irregularities too small for the bacteria to fit into them reduce the contact area and binding (Edwards,KJ and Rutenberg,AD. 2001).

Biomaterial surface hydrophobicity and charge have been suggested to be important factors in bacterial adhesion. Metal surfaces have a high surface energy and are negatively charged and hydrophilic as seen from water contact angles. Polymers such as UHMWPE are less electrostatically charged, hydrophobic and have low surface energy. Bacteria adhere differently to materials with different hydrophobicities depending on the hydrophobicities of both bacteria and material surfaces. Hydrophobic material surfaces attract bacteria with hydrophobic surfaces, and hydrophilic material surfaces attract microbes with hydrophilic surfaces.

The atomic structure of the surface presents energy profiles or available binding sites for bacteria. Most substrata have a negative charge, but it can vary depending on pH, inflammation and tissue damage caused by surgery, trauma, infection, and corrosion, which alter pH and charge. Usually, electrostatic interactions create repulsion between bacteria and substratum because surfaces of bacteria and material tend to both have a negative charge. Surface texture, manufacturing processes, trace chemicals and debris, and by ionic and glycoproteinaceous constituents from the host environment (conditioning film), binding sites are further modified regarding polymer and metal surfaces (Gristina,AG. 1987).

Environmental conditions for opportunistic bacteria to start infections are established through substratum disruption by trauma, wear, corrosion, toxins, viral effects, bacterial mechanisms or biosystem chemical degradation. Implant materials are surrounded by an immuno-incompetent, fibro-inflammatory zone, where stimulation of cellular immune responses results in superoxide radical and cytokine-mediated tissue damage which further increases susceptibility to implant infection or aseptic loosening (Gristina,AG. 1987, Gristina,AG. 1994, Tang,L and Eaton,JW. 1995). Material mediated inflammatory processes may even cause degradation of the material itself (via oxidative products) and thus, increase susceptibility to infection (Tang,L and Eaton,JW. 1995).

2.3.4.6 Environmental properties

Environmental factors such as temperature, time of exposure, bacterial concentration, the presence of antibiotics, chemical treatment and flow conditions affect bacterial adhesion. Flow conditions strongly influence the number of attached bacteria (Isberg,RR and Barnes,P. 2002), the biofilm structure and performance (Stoodley,P, et al. 1999). Higher shear rates result in greater detachment forces that decrease the number of attached bacteria and make the biofilm denser and thinner (Chang,HT, et al. 1991).

In ligand/receptor-mediated specific bacterial adhesion, the number of bonds that can form will be a function of ligand and receptor densities (Hubble,J, et al. 1996, Mascari,L, et al. 2003). If each bond requires a specific force to break it, the number of bonds between a microbe and a surface determine the shear stress that the attached bacterium will be able to resist. An optimum flow rate for bacterial attachment has been found, describing the balance between the speed of the delivery and the force acting on attached bacterium

(Liu,Y and Tay,JH. 2002). For *S.aureus*, in the case of a higher number of receptors/cell, between shear rates 50-300 $s^{-1,}$ adhesion to collagen coated coverslips increases and then decreases for shear rates greater than 500 s^{-1} (Mohamed,N, et al. 2000). Thus, binding is increased, up to a certain shear rate (Mohamed,N, et al. 2000). Greater mass transport means that more bacteria are delivered into proximity of the surface in a given time unit. Also, the binding capacity of the bacterial surface is altered, as new adhesive surface proteins and structures come into play. In this way, microbes can form stronger bonds with the surface to withstand surrounding flow and adhere in a different way than in static conditions. Above border values, with higher shear rates, bacterial adhesion, and biofilm retention is decreased, leading to detachment of bacteria and biofilms from the surface (Katsikogianni,M, et al. 2006).

It is also reported that suspended bacteria can respond to shear by altering their growth rate, morphology, bacteria size/density and metabolism (Liu,Y and Tay,JH. 2002). Additional interactions are progressively formed after the initial bacterial attachment in flow conditions. Up to a maximum value, the shear stress required to generate detachment increases with incubation time (Ming,F, et al. 1998).

The ionic strength (electrolytes) and pH value affect bacterial adhesion by changing surface characteristics of both the bacteria and the materials (hydrophobicity-charge) (Katsikogianni,M and Missirlis,YF. 2004). The presence of antibiotics decreases bacterial adhesion depending on bacterial susceptibility and antibiotic concentration.

Serum or tissue proteins (e.g. albumin, fibronectin, fibrinogen, laminin, denaturated collagen) may promote or inhibit bacterial adhesion in binding to substrata and to the bacterial surface, or being present in the liquid medium during the adhesion process. For the latter situation, most of the proteins have been shown to inhibit bacterial adhesion (Brokke,P, et al. 1991), possibly by their association with the bacterial cell surface, the material surface or both. Proteins may change bacterial surface physicochemical characteristics and in this way alter the adhesion process (An,YH and Friedman,RJ. 1998, Pascual,A, et al. 1986). Usually, the bindings between bacteria and proteins are specific ligand-receptor interactions. ECM protein Fibronectin (Fn) has been shown to promote binding of *S.aureus* (Kuusela,P, et al. 1985) to the substratum and play a major role in foreign body infections (Vaudaux,P, et al. 1984). Regarding the effect of Fn on *S.epidermidis* binding, controversial results exist (Herrmann,M, et al. 1988). Albumin has clearly inhibited bacterial adhesion to the polymer (Hogt,AH, et al. 1985, Pascual,A, et al. 1986), ceramic (Gibbons,RJ and Etherden,I. 1983), and metal (An,YH, et al. 1996, An,YH, et al. 1995) surfaces. This inhibition of bacterial adhesion by albumin is thought to involve binding to bacterial cells or alteration of the substratum to be more hydrophilic (Fletcher,M and Marshall,KC. 1982). A serum protein fibrinogen has been reported to enhance adhesion of bacteria and especially staphylococcal adhesion to biomaterials (Cheung,AL and Fischetti,VA. 1990, Herrmann,M, et al. 1988). Serum and plasma have shown inhibitory effects on adhesion, mainly due to albumin, while IgG and Fn are less efficient (Pascual,A, et al. 1986, Paulsson,M, et al. 1993). Inserted implants encounter different cells and tissue elements such as platelets *in vivo*. Wang *et al*. reported that platelets mediated *S.epidermidis* adhesion onto hydrophobic PE surfaces (Wang,IW, et al. 1993).

In conclusion, the acceleration of bacterial metabolic processes that result in growth and biofilm formation after the contamination of a biomaterial surface may be explained by several factors. Increased chemical activity (catalysis), the creation of new molecules and the presence of free ions released from the biomaterial or as contaminants on the biomaterial surface play a role here (Gristina,A, et al. 1987).

2.3.5 Biofilm formation

Definition and mechanisms

A biofilm has been defined as a layer of prokaryotic or eukaryotic cells, anchored to a substratum (biological or non-biological) and embedded in an organic matrix of biological origin (Bos,R, et al. 1999). Even though biofilms are often linked with surface attachment, they can also form at interfaces of spatially distinct microenvironments or as suspended aggregates of free-floating cells. These aggregated masses may present features similar to those of a typical surface-associated biofilm (Costerton,J. 2007, Hall-Stoodley,L, et al. 2012). Hall-Stoodley *et al.* suggested the definition of a clinically relevant biofilm as: 'aggregated, microbial cells surrounded by a self-produced polymeric matrix, which may contain host components' (Hall-Stoodley,L, et al. 2012).

As structurally complex dynamic systems, biofilms have characteristics of both multicellular eukaryotic organisms and diverse ecosystems (Hall-Stoodley,L, et al. 2004). Bacteria in biofilms communicate, collectively regulate many physiological activities and share metabolic pathways (Donlan,RM. 2002, Dunne,WM,Jr. 2002). Due to the lack of proper diagnostic criteria of biofilm infections, the role of biofilms in human disease is often not clear. Parsek and Singh (Parsek,MR and Singh,PK. 2003) have proposed four criteria for infections of biofilm origin. First, the pathogenic bacteria should be surface associated or adherent to a substratum. Second, a direct examination should reveal bacteria in clusters embedded in a matrix of bacterial or host constituents. Third, the infection should be localized. Fourth, the infection should be resistant to antibiotic therapy despite the antibiotic sensitivity of the constituent planktonic organisms.

Planktonic or suspended cells are primarily single cells growing in suspension. Sessile cells are attached to surfaces but may be dynamic and move according to the latest evidence (Hall-Stoodley,L, et al. 2004). Adhesion to surfaces has been shown to induce expression of genes that result in the conversion of cells from planktonic single-cell free-swimming cells into a complex multicellular and multilayered biofilms on a surface with bridging connections between bacterial cells (Heilmann,C, et al. 1996, Mack,D. 1999). This change of a phenotype leads to resistance to antibacterial agents, phagocytes, and antibodies. Due to altered bacterial metabolism, genetic exchange and slimy barrier mechanism of biofilm, microbes become highly resistant to antibiotics in biofilms. Much larger concentrations of antibiotics are needed to kill bacteria in biofilm than planktonic bacterial cells.

Biofilm matrix consists of bacterial cells, exopolysaccharides, proteins, lipids, teichoic acids, extracellular DNA (eDNA) released from dying cells, and other polymers

(Arciola,CR, et al. 2012). The most important component of the ECM produced by staphylococci is exopolysaccharide PIA (Mack,D, et al. 1996a), although evidence exists that *in vivo* and *in vitro* staphylococcal biofilms can form without PIA (Rohde,H, et al. 2007). In the abundance of PIA, other matrix components substitute the missing exopolysaccharide.

PIA is found in biofilm producing Gram-negative bacteria and in many staphylococci. Its synthesis is controlled by the intercellular adhesion (icaADBC) locus that is found in *S.aureus* and in *S.epidermidis* as well as in several other Staphylococcus species (Heilmann,C, et al. 1996, Vuong,C, et al. 2004b). In *S.aureus* and *S.epidermidis,* the expression of ica operon and the production of biofilm have been shown to depend on environmental conditions, such as culture media composition and supplementation, ethanol, salt stress, iron limitation and anaerobiosis (Arciola,CR, et al. 2012). There is even evidence that *S.aureus* can modulate its metabolism switching from the production of a proteinaceous to an exopolysaccharide biofilm matrix, depending on the external stimuli (Houston,P, et al. 2011, Vergara-Irigaray,M, et al. 2009).

Teichoic acids on the cell surfaces of Gram-positive bacteria are negatively charged and contribute to biofilm formation in staphylococci, probably by interacting with other surface polymers and taking part to protein attachment (Gross,M, et al. 2001). *S.aureus* and *S.epidermidis* surface proteins associated with biofilm formation and function have been characterized and include protein A, biofilm-associated protein (Bap) and fibronectin-binding proteins FnbpA and FnbpB (Joo,HS and Otto,M. 2012). It has been recently indicated that *in vitro* biofilm formation of *S.aureus*, particularly MRSA may depend more on eDNA and proteins, whereas PIA may play a significant role in *S.epidermidis* and methicillin-susceptible *S.aureus* (Pozzi,C, et al. 2012).

Biofilm formation can be devided into four stages in the biofilm life cycle. The first step involves bacterial adhesion to a surface. Secondly, microcolonies are formed, and the third stage describes biofilm maturation. In the final detachment stage, the cells leave the biofilm structure to contaminate other surfaces (Abdallah,M, et al. 2014). As biofilm formation starts with bacterial adhesion to the substratum, there is overlapping of the factors that influence bacterial adhesion and biofilm formation even though they are two distinct phenomena. The function and appearance of biofilms in various environments may be different, but in the end, they originate from the same sequence of events (Escher,A and Characklis,W. 1990, van Loosdrecht,MC, et al. 1990). The development of biofilm is a dynamic process involving attachment, adhesion, aggregation, growth cycles (proliferation), moving over surfaces, detachment/dispersal, and reattachment (Hall-Stoodley,L, et al. 2004).

After initial attachment to the surface, bacteria adhere specifically with receptor-ligand and cell-to-cell molecular connections. Microcolonies of one or several species are formed as multilayered cellular proliferation proceeds, and more bacteria adhere to sessile bacteria, conditioning film or slimy extracellular polysaccharide matrix associated with the surface. Bacteria secrete EPS, main constituents of the structural matrix surrounding the bacterial cells (Hoiby,N, et al. 2011). This matrix acts as a scaffold that stabilizes the three-dimensional structure of a complete biofilm in the maturation phase. In this phase, physiological changes occur within the biofilm, including regulation of pili, flagella, and exopolysaccharides. With the altered dormant phenotype, metabolism of sessile microbes

slows down, and proteins produced and expressed change as well as genes regulated and expressed. The maturation stage is mainly controlled by accessory gene regulator (agr) locus and the quorum sensing (QS) system. Otto *et al*.(Otto,M. 2001, Vuong,C, et al. 2000) reported for the first time the importance of the agr system in biofilm formation in both *S.aureus* and *S.epidermidis*. Communication and signaling between bacteria in the biofilm, QS, has been suggested to be an essential element of regulation of biofilm life cycle, biofilm formation and virulence (Periasamy,S, et al. 2012, Vuong,C, et al. 2000, Vuong,C, et al. 2003, Vuong,C, et al. 2004a). Sessile bacteria can signal with each other and with closing planktonic microorganisms.

The bacterial detachment from biofilm can be an active process (dispersal), a passively induced mechanical process (through fluid shear) or a chemical process (through agents that dissolve EPS matrix) (Hall-Stoodley,L, et al. 2004). The agr system and proteases control this detachment phase. Three distinct biofilm dispersal strategies have been found: swarming/seeding dispersal, in which individual cells are released into the surrounding fluid or the surrounding substratum; clumping dispersal in which aggregates of cells are shed as clumps or emboli; and surface dispersal in which biofilm structures move across the surfaces. Then, they can start colonization and biofilm formation at other locations (metastasis). This can be the beginning of late hematogenous infections in the human body at other sites, distant from the primary infection locus.

In addition to available nutrients and mass transfer due to fluid shear, biofilm heterogeneity can also be maintained through the production of diffusible detachment factors, which cause localized detachment (Hunt,SM, et al. 2003). When the population of microbes exceeds a certain level, and the surface becomes overpopulated, parts of biofilm detach and move elsewhere to find new growth platforms. With motile bacteria, assisting dispersal, upregulation of motility begins in the center of the biofilm mushroom caps. Biofilm dispersal is thought to depend on cell-cell disruptive factors, identified to be primarily surfactants controlled by QS. In QS increased cell density triggers changes in gene expression and it has been associated with a significant number of developmental processes including the regulation of biofilm formation and maturation.

The structure of biofilms changes through mass transfer, genetic regulation, and selection depending on available nutrients and environmental conditions. In fast-moving waters, biofilms form filamentous microcolonies called streamers and in calm waters mushroom-like structures. Biofilms grown in flow conditions show viscoelastic (elastic: solid-like and viscous: liquid-like) behavior. They can withstand the temporary periods of rapidly changing shear stresses common in marine and river environments (Klapper,I, et al. 2002, Stoodley,P, et al. 2002). Observation of biofilms growing in nature and the results of *in vitro* work show, that both the environment and the genome influence biofilm formation and development (Hall-Stoodley,L, et al. 2004).

2.3.6 Race for the surface

The competition between bacterial cells and host tissue cells on an available surface for colonization always occurs when an implant is inserted in the human body. Anthony

Gristina referred to this competition as "the race for the surface" (Gristina,AG. 1987). A bare biomaterial surface is first coated with ECM and plasma proteins, as a conditioning film forms. In the next step, the race for the available surface begins, which determines whether the host cells or microbes adhere first to the substratum. If host tissue cells win the race and colonize the surface, the closing bacteria will face integrated viable host tissues with efficient defense systems and mechanisms. In this way, bacterial colonization would be less likely to occur, and an infection could be prevented. If bacterial cells adhere to the surface before tissue cells, an infection is more likely to establish as primary colonizers can recruit other bacteria to the surface and host defense systems can be compromised due to the formation of immuno-incompetent microzone. This axiom means that implant surfaces with excellent biocompatibility and antibacterial properties at the same time would be the optimal choice for the prevention of device-related infections. Such surfaces would allow human tissue cells to adhere and grow on them but would inhibit bacterial adherence and biofilm formation. The race for the surface concept has been experimentally evaluated by Subbiahdoss *et al*. (Subbiahdoss,G, et al. 2009) and the work in this field continues (Perez-Tanoira,R, et al. 2017, Yue,C, et al. 2015, Zhao,B, et al. 2015).

2.3.7 Methods for investigating bacterial adhesion and biofilm formation

2.3.7.1 Static and dynamic testing of bacterial adhesion

Bacterial adhesion studies can be done either in static conditions or dynamic flow systems. A surface is overlaid or overflown with a suspension of bacteria for a predetermined period. Microbes can be visualized in real time, or not adherent microbes are separated by rinsing or centrifugation, after which the adhered bacteria are visualized and counted. In static experiments usually commercially available flat surfaces like plastic culture tubes, petri dishes, tissue culture plates or chemotaxis chambers function as platforms for bacterial adhesion. Otherwise, microbes adhere onto substrata placed into these petri dishes, culture tubes and culture plates (An,YH and Friedman,RJ. 1997). Under dynamic flow conditions, bacterial adhesion can be tested by several methods. A flow cell perfusion model (Yu,JL, et al. 1996), rotating disc apparatus (Rutter,PR and Abbott,A. 1978), parallel plate flow chamber (Rutter,P and Leech,R. 1980), radial flow chamber (Duddridge,JE, et al. 1982) or Robbins device (McCoy,WF, et al. 1981) have been used. In these systems, an oriented fluid flow brings bacteria to the material surfaces. Real-time observation is possible with certain experimental settings (Yu,J, et al. 1994). Controlled shear and mass transport, a high data density in time and no air-liquid interface passages over the adhering bacteria are the advantages of flow systems (Busscher,HJ and van der Mei,HC. 1995).

Microscopy can be used for counting and morphological analysis of adherent bacteria, as in light-, image-analyzed epifluorescence-, scanning electron-, confocal laser scanning, and atomic force microscopy. Viable bacteria counting methods such as Colony Forming Unit (CFU) plate counting, radiolabelling, and staining are useful in the assessment of the viability of the microbes. Other direct and indirect methods such as spectrophotometry,

Coulter counter, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and biochemical markers add to the repertoire of possible examination tools.

Light microscopy is a basic method for bacterial enumeration and observation. It is used to observe bacteria and biofilm directly (Trulear,MG and Characklis,WG. 1982) or through a histologic section, with paraffin-embedded biofilms that are cut into histologic sections (Chang,CC and Merritt,K. 1992).

Most reliable tool for the observation of smooth and opaque, like metal, plastic or ceramic surfaces is Image-analyzed epifluorescence microscopy (IAEFM), based on fluorescence stained samples (An,YH, et al. 1995). When combined with epifluorescence or fluorescence microscopy, immunofluorescence stainings can be used to assess surface attached bacteria. Specific antibodies are produced by immunization of animals and are conjugated with fluorescence stains. Even in the presence of other strains or species, it is possible to enumerate and identify specific attached bacteria (An,YH and Friedman,RJ. 1997). Epifluorescence microscopy has several advantages. It is very quick, reducing the time required for visual counting by 85% (Sieracki,ME, et al. 1985) and it is suitable for large sets of samples. Additionally, epifluorescence microscopy can reduce the possibility of operator bias, and it makes direct observation and enumeration possible for attached bacteria on opaque surfaces (An,YH and Friedman,RJ. 1997). With individual fluorochromes, it allows differentiation between live and dead bacterial cells on the surface. Image analysis systems have been used for determining the number of adherent cells, area coverage, and biovolume of attached cells as well as the real-time evaluation of attachment, detachment, and growth on the surfaces.

2.3.7.2 Biofilm examination

Infected implants or infected or dead bone surfaces can be the sources of biofilms harvested for *in vivo* experiments (Vaudaux,P, et al. 1989). These biofilm samples represent better conditions prevailing in the human body but are more complicated due to the presence of proteins, tissue cells or debris mixed in a biofilm. Implants together with adherent bacteria can be inserted into animals for biofilm studies *in vivo* and left in animal tissues for days, weeks or even months before sacrificion of animals for biofilm evaluation. Bacteria can also be injected into the implant site after implantation (An,YH and Friedman,RJ. 1997).

To recognize bacterial biofilm the adherence tube test is applied (Christensen,GD, et al. 1982). In this approach, biofilm lining the inner surface of the tube is monitored. It has been used widely as a screening method for slime-producing bacteria. A turbulent flow system, rotating disk reactor and radial flow reactor have been used to study biofilm *in vitro* in dynamic conditions (An,YH and Friedman,RJ. 1997). Different methods are available for biofilm evaluation and quantification ranging from microtiter plate attachment testing to biofilm reactors and from microscopic evaluation to mathematical evaluation of images acquired by Confocal Laser Scanning Microscopy (CLSM).

The thickness of the biofilm can be calculated using light microscopy or scanning electron microscopy (SEM). Then the dry biofilm mass can be utilized with the thickness

and surface area measurements to obtain the density of the biofilm sample (An,YH and Friedman,RJ. 1997). Directly or through histological sections, light microscopy enables biofilm observation morphologically. The structural properties of a biofilm are viewed with SEM and transmission electron microscopy (TEM). One must consider that SEM and TEM require sample preparation. Morphologically CLSM is superior in its ability to examine biofilm *in situ* in hydrated conditions; also three-dimensional structures are visualized with optical sectioning of the samples (Lawrence,JR, et al. 1991, Qian,Z, et al. 1996). CLSM uses fluorescent molecular probes and laser beams with a broad range of applications in biomedical research. The distribution of bacteria or the thickness of the biofilm and the viability of bacterial cells can be assessed. Environmental scanning electron microscopy (ESEM) enables the observation of wet samples what makesthe sample preparation minimal. There has been interest in applications of ESEM in biomaterial sciences (Manero,JM, et al. 2003).

Various methods provide variety of information on biofilm content. The method by Christensen et al. enables studies of the relative production of slime by different strains and species of coagulase-negative staphylococci. Additionally, evaluation of effects of various conditions such as antibiotic exposure on slime production (Christensen,GD, et al. 1985) can be evaluated. Extracellular slime substance of *S.epidermidis* has been analyzed with an immunochemical method developed by Kotilainen et al. (Kotilainen,P, et al. 1990). Radiolabelling has been used to measure the biofilm quantitatively (Hussain,M, et al. 1992) by growing coagulase-negative staphylococci in a medium containing [^{14}C] glucose, which leads to the labeling of the extracellular polysaccharide.

There are difficulties in studying surface-associated bacteria in biofilms compared to planktonic microbes. More work and time are needed to culture bacteria as biofilms, as heterogeneity of spatial distribution leads to the formation of localized zones, where both physiological conditions and cellular physiologies vary widely over subtle distances. Moreover, the mass transfer (diffusion and flow through biofilm) and fluid forces (shear and drag) can have influence on biofilm in bacterial surface cultures (Hall-Stoodley,L, et al. 2004).

2.3.8 Fighting joint implant infections

2.3.8.1 Antibacterial biomaterials

An anti-infective biomaterial is defined as a biomaterial, which has the property to prevent or, reduce the infection caused by pathogens of any type (generically including viruses, bacteria, fungi, protozoa or other pluricellular pathogens such as helminths) (Campoccia,D, et al. 2013b). In recent years, biomaterial science has taken significant leaps in design and development of anti-infective biomaterials, which is reflected in many research papers related to these topics. With current improvement of aseptic surgical techniques and procedures, adequate preparation of the patient, hygienic protocols, laminar air-flow, good antibiotic prophylaxis and postoperative wound care it seems that anti-infective biomaterials offer a rational and desirable solution in the prevention of medical device-related infections and could lower the risk of these complications further.

By definition, antibacterial biomaterials have bactericidal properties, inhibit bacterial growth or hinder bacterial colonization. Various strategies are available in the development of a biomaterial with antibacterial properties: 1) bacteria-repelling and antiadhesive surfaces; 2) intrinsically bioactive materials with antibacterial properties; 3) bioactive antibacterial coatings; 3) biomaterials delivering antimicrobials; 4) nanostructured materials and 5) bioactive molecules interfering with the production of bacterial biofilm (Campoccia,D, et al. 2013a).

The concept of bacteria-repelling and antiadhesive surfaces has a long time been related to the adsorption of molecules that increase hydrophilic properties of the material surface and compete with the interaction between bacteria and host matrix proteins that cover the implant surface. Heparin has been used in anti-adhesive coatings due to its high hydrophilic properties (Arciola,CR, et al. 1993, Arciola,CR, et al. 1994). Other examples are coatings based on hydrophilic polymeric brushes based on poly (ethylene glycol) (PEG) or poly (ethylene oxide) (PEO) (Neoh,KG and Kang,ET. 2011). However, so far it has been shown that, generally, negatively charged surfaces, super hydrophobic surfaces, super hydrophilic surfaces, and nm-scale surface roughness all reduce bacterial adhesion. Some positively charged surfaces also have antimicrobial properties, when appropriate materials are coated on a substrate. The DLC and the DLC polymer hybrid coatings tested in this thesis belong to this group of bacteria-repelling and antiadhesive surfaces.

Intrinsically antibacterial materials show antibacterial activity in bulk form without modifications. In this group can be categorized several metals (e.g. silver, zinc, and copper), some polymeric materials (e.g. chitosan and its derivatives) and different bioactive glasses. Rarely antimicrobial metals are used as bare bulk biomaterials. Instead, they are utilized in nanocoatings, in doped solid or hydrogel materials, in the formulation of bioactive alloys and glasses and in form of micro-and nanoparticles. Polymers that contain molecules with antimicrobial properties in their molecular structure belong to the group of intrinsically antibacterial materials (Neoh,KG and Kang,ET. 2011, Tan,H, et al. 2012).

Bioactive antibacterial coatings deliver anti-infective properties without compromising the essential bulkmaterial characteristics. They can be based on different concepts. Antimicrobial molecules (e.g. triclosan, chlorhexidine) can be fixed on the material surface by grafting; polymer coatings can possess functional groups with bactericidal activity (e.g. tertiary amines, N-alamines) or polymer coatings may release nitric oxide. Also, polymer coatings releasing reactive oxygen species can be used or coatings made of substrates that become bactericidal after a process of photoactivation. Thin inorganic or organic, single or multilayer, films consisting of or delivering antimicrobial molecules can be considered. Finally, nanotechnology can be utilized to produce bactericidal nanostructured surfaces or coatings with nanostructured materials (Campoccia,D, et al. 2013a).

In the case of using biomaterials delivering antimicrobial agents, the antimicrobial substances can either be incorporated in the bulk or in the coating of a biomaterial. Several methods exist, such as mixing of the substances to the ingredients during the production (e.g. in setting cement), binding of substances covalently to functionalized polymeric

coatings or incorporation of substances in self-assembling mono/multilayer organic coatings. An issue of concern has been the development of antibiotic resistance with sub-inhibitory concentrations of antibiotics, as the antibiotics released by antibiotic-loaded biomaterials diminish over time. Also, systemic toxicity has been noted in patients having antibiotic-loaded orthopedic implants (Campoccia,D, et al. 2010). Nevertheless, antibiotic impregnated biomaterials are of great value in certain procedures, such as two-stage revision operation of the orthopedic joint prosthesis.

Additionally, systemic and local toxicity (especially with large implant surfaces and in the proximity of central nervous system) associated with the release of metal ions such as Ag+ silver have guided the research community to search new tools and antimicrobial substances. This has led to introduction of antimicrobial peptides (AMPs) as local, rapid, selective and broad-spectrum molecules that can be delivered from biomaterials (Hancock,RE and Sahl,HG. 2006). Obstacles challenging the utilization of these peptides in biomedicine include potential local toxicity, allergy, susceptibility to proteases and pH changes and the high cost of peptide production. While clinical trials are under way, interest in AMPs is increasing, and new synthetic compounds aiming to minimize drawbacks and maximize the bactericidal possibilities are produced (Kang,SJ, et al. 2012). One strategy to prevent implant infections with biomaterial science is to produce biomaterials, in which anti-adhesive and antimicrobial coatings are combined. This is seen in multilayer films constructed by assembling layer-by-layer heparin and chitosan, and covalent conjugation of AMPs immobilized onto a hydrophilic polymer (Fu,J, et al. 2005, Gao,G, et al. 2011).

As nanostructural features of material surfaces can alter the 3D conformation of adsorbed proteins, nanostructured materials can have an impact on host adhesins and conditioning film (Montanaro,L, et al. 2008). Antimicrobial nanoparticles (NPs) are gaining interest in the field of nanotechnology, and thin surface coatings can be developed at a nanoscale level. NPs are described as regularly or irregularly shaped particles with at least a dimension smaller than 100 nm (Campoccia,D, et al. 2013a). Even though metal oxides and carbon-based NPs have shown promising antibacterial activity, the current knowledge of safety and toxicological aspects of nanomaterials require careful evaluation before routine clinical use of these materials. While physicochemical properties and functional groups on material surface guide bacterial adhesion, the morphology of the surface influences the outcome of the adhesion process and possibly cell metabolism (Campoccia,D, et al. 2013a). Specially patterned surfaces can direct the alignment and the spatial distribution of bacterial cells. Lowest adhesion of Gram-negative and Gram-positive bacteria was seen *in vitro* on smooth surfaces on nanometric levels (Mitik-Dineva,N, et al. 2009).

Many active anti-biofilm substances have been identified that can be grafted on biomaterial surfaces or released by adequate systems/coatings (Arciola,CR, et al. 2011). Their molecular components have different modes of action. For example, enzymes are capable of selective degradation of the extracellular polymeric substance of biofilm (e.g. Dispersin B) ; other bactericidal molecules have abilities to destroy even metabolically quiescent bacterial cells within biofilms (e.g. certain AMPs). Molecules interfering with the QS system and inducing biofilm dispersion (e.g. furanones) and molecules downregulating the expression of biofilm extracellular polymeric substances (e.g. N-

acetylcysteine) or any way reducing biofilm metabolism (e.g. hamamelitannin) (Campoccia,D, et al. 2013a) have also been under evaluation. The differences between bacterial species and strain types of the same species and adaptation of the bacterial genome are among the few questions debated regarding clinical applications of bioactive molecules interfering with the production of bacterial biofilm. Promising results have been reported in studies where anti-biofilm strategies have been combined with the regular delivery of antimicrobials (Donelli,G, et al. 2007).

2.3.8.2 Host natural response: antimicrobial peptides

2.3.8.2.1 Overview

The AMPs are an ancient and significant part of the host defense and immunity in invertebrates, vertebrates, and plants (De Smet,K and Contreras,R. 2005). The two most important antimicrobial peptide families in humans and other mammals are defensins and cathelicidins. AMPs are short peptides with broad-spectrum activity against a broad range of bacteria, fungi, viruses, parasites and even cancerous cells (Wang,G. 2014). They are highly reactive to infectious agents and innate immunostimulatory molecules and exhibit immunomodulatory properties. AMPs are chemotactic for leukocytes and nonimmune cells at nanomolar concentrations (Auvynet,C and Rosenstein,Y. 2009). They work in a cooperative way by leading effector cells to the site of inflammation, modulating the local immune response and directing the order of appearance of the different players in different scenarios (Auvynet,C and Rosenstein,Y. 2009). AMPs indirectly support chemotaxis by inducing the secretion of chemokines (Scott,MG, et al. 2002, Tjabringa,GS, et al. 2003). Antimicrobial peptides can be both pro- and anti-inflammatory depending on the situation as they protect the host from microbial attacks and at the same time hinder excessive immune response (Biragyn,A, et al. 2008, Semple,F and Dorin,JR. 2012).

In May 2014, the current AMP database contained over 2400 AMPs and proteins, with 98.5% from living prokaryotic and eukaryotic organisms (http://aps.unmc.edu/AP/main.php). It is reported that approximately 10% of available AMPs are anionic peptides, and 90% are cationic peptides. With the progress of antibiotic resistance to multiple antimicrobial agents, new therapeutical tools are needed. Microbial resistance against AMPs is rare. Therefore, AMPs are considered promising in the search for novel therapeutical applications (Batoni,G, et al. 2016, Martin,L, et al. 2015). In diagnostics, the potency of AMPs has also been noted (Deirmengian,C, et al. 2014, Deirmengian,C, et al. 2015, Matsen Ko,L and Parvizi,J. 2016). So far high costs and some setbacks in clinical trials and commercializing attempts have delayed bringing AMPs to the pharmaceutical market. Nevertheless, substantial progress is made in response to increased antibiotical resistance and the emergence of new pathogens (Fox,JL. 2013, Gordon,YJ, et al. 2005).

2.3.8.2.2 Defensins

Defensins are small, cationic and cysteine-rich peptides secreted in many species including humans and other mammals, fishes, birds, filamentous fungi, and plants. They are the largest group of the AMP family and in humans classified into two subgroups (α- and β-defensins) based on unique amino acid sequences and disulfide connectivities. The antimicrobial action of granulocytes, mucosal host defense in the small intestine and epithelial host defense in the skin and elsewhere have been associated with the defensins. *In vivo* experiments report that defensin molecules are important in antimicrobial defense (Morrison,G, et al. 2002, Salzman,NH, et al. 2003, Taylor,K, et al. 2008, Wilson,CL, et al. 1999).

Microbial signals, developmental signals, cytokines and some neuroendocrine signals mediate defensin synthesis and release (Ganz,T. 2003). Antimicrobial activity of defensins has been linked to permeabilization of target membranes; several theories exist, but the exact mechanism is unclear (Brender,JR, et al. 2012, Ganz,T. 2003, Lichtenstein,A. 1991). There have also been reports about specific microbial resistance against antimicrobial peptides/defensins (Joo,HS and Otto,M. 2015, Lai,Y and Gallo,RL. 2009, Peschel,A and Sahl,HG. 2006). However, as antimicrobial peptides are human body´s natural antibiotics preserved through evolution; it is thought that this resistance is rare compared to that prevailing against common antibiotics. Further studies will bring more valuable information regarding this subject.

The human α-defensins (hADs) 1-4 are mainly found in neutrophils but also expressed in natural killer (NK) cells, monocytes, and some T lymphocytes subsets. hADs 5 and 6 were detected first in Paneth cells of the small intestine and additionally both in respiratory and gynecological tracts.

The human β-defensins (hBDs) 1-4 are mainly secreted by a large variety of mucosal epithelia such as those lining urogenital, gastrointestinal and respiratory tracts (De Smet,K and Contreras,R. 2005). hBDs have crucial functions in innate immunity as the first line of defense and are associated with various infectious and inflammatory states. They have broad-spectrum of antimicrobial activity against Gram-negative and Gram-positive bacterial strains, fungi, some parasites and enveloped viruses and low drug resistance (Semple,F and Dorin,JR. 2012). hBDs are produced either constitutively or in response to infection and tissue injury (Dhople,V, et al. 2006, Wang,G. 2014).

hBD-1 was first isolated from the hemofiltration of patients undergoing dialysis treatment (De Smet,K and Contreras,R. 2005). hBD-1 is synthesized by epithelia that are in direct contact with the environment or microbial flora (lung, mammary gland, salivary gland, kidney, pancreas and prostate epithelia) (Bensch,KW, et al. 1995). hBD-2 has been associated with similar locations although it has also been found in skin, leukocytes, urogenital system, gut and bone marrow. hBD-2 was first isolated from a psoriatic skin lesion (Harder,J, et al. 1997). hBD-1 is reported to be synthesized constitutively while hBD-2 expression has been shown to be upregulated by exposure of epithelial cells to lipopolysaccharide (LPS) or pro-inflammatory cytokines (tumor necrosis factor alpha (TNF-α) or interleukin 1 beta (IL-1β)) (De Smet,K and Contreras,R. 2005, Harder,J, et al. 1997). hBD-3 has been detected in epithelia and non-epithelial cells in the heart, liver, skeletal muscle, leukocytes, and placenta (Garcia,JR, et al. 2001a, Harder,J, et al. 2001,

Pazgier,M, et al. 2006). It was first isolated from human lesional psoriatic scales (De Smet,K and Contreras,R. 2005). hBD-3, like hBD-2, and is also induced by inflammatory stimuli and contact with bacteria.

Three other β-defensin genes (hBD-4-6) were found with the Basic Local Alignment Search Tool (BLAST) bioinformatics and functional genomic analysis in the region 8p23 of human chromosome (Garcia,JR, et al. 2001b, Yamaguchi,Y, et al. 2002). These defensins are less known; hBD-4 is present in testis, epididymis, lung tumor tissue and gastric epithelial cells (Auvynet,C and Rosenstein,Y. 2009, Shestakova,T, et al. 2008).

Yamaguchi et al. 2002 suggested classification of the hBDs in two groups: the epididymis-specific isoforms (hBD 4-6) and the other isoforms (hBD1-3) (Yamaguchi,Y, et al. 2002). 28 new hBD genes were discovered by using a computational search tool based on hidden Markov models in combination with BLAST (Schutte,BC, et al. 2002).

It has been suggested that the influence of β-defensins might reach beyond their antimicrobial activity to roles in development, wound healing, fertility, and cancer (Semple,F and Dorin,JR. 2012). In literature, the defensins have been linked to antitumor activity (Lichtenstein,A, et al. 1986), stimulation of cell proliferation (Murphy,CJ, et al. 1993), interference with signal transduction pathways (Charp,PA, et al. 1988), chemoattraction of immune cells (Territo,MC, et al. 1989) and stimulation of cytokine and adhesion molecule expression (Chaly,YV, et al. 2000).

2.3.8.2.3 hBD-3

The human β-defensin-3 (hBD-3) expression is influenced by bacterial-derived molecules, cytokines, and chemokines produced by the immune system or damaged cells, which reflects the time and site-specific, on-demand utilization of this defensin in various scenarios considered hostile for the human body. Interestingly, hBD-3 has a broader spectrum of antimicrobial activity than hBD-1 and hBD-2, which are effective against Gram-negative bacteria and some fungi. hBD-3 is active also against Gram-positive bacteria and even kills or inhibits the growth of the opportunistic pathogenic yeast, *Candida Albicans* and some multidrug-resistant clinical isolates such as vancomycin-resistant *Enterococcus faecium* (VER) and MRSA. hBD-3 was initially isolated from lesional psoriatic scales and has been cloned from both keratinocytes and lung epithelial cells. It was found when human epithelial cells were screened for endogenous *S.aureus*-killing factors (Dhople,V, et al. 2006, Feng,Z, et al. 2005, Harder,J, et al. 2001, Lee,JK, et al. 2013). hBD-3 acts in a salt-insensitive manner what is unusual for defensins (Harder,J, et al. 2001). High net charge and hydrophobicity of hBD-3 have been linked to its antimicrobial activity (Kluver,E, et al. 2005).

No cytotoxicity with hBD-3 has been recognized, which has been explained by differences in the membrane structures of microbial cells and eukaryotic cells and different affinities of hBD-3 to them. While human eukaryotic cells have neutral zwitterionic cholesterol and phospholipids in their outer membranes which hBD-3 binds weakly, bacterial cell membranes have anionic phospholipids that cationic defensins find attractive (Brender,JR, et al. 2012).

hBD-3 is chemotactic to cells important in septic and aseptic loosening of the implant, such as neutrophils, monocytes, and macrophages, through binding to chemokine receptor type 2 (CCR2) (Garcia,JR, et al. 2001a, Rohrl,J, et al. 2010). hBD-3 has also been suggested to bind chemokine receptor type 6 (CCR6) (Wu,Z, et al. 2003), but this is still disputed (Soruri,A, et al. 2007). hBD-3 activates monocytes and myeloid dendritic cells via Toll-like receptors 1/2 (TLR1/2) (Soruri,A, et al. 2007) and can also bind TLR9 intracellularly after penetration of the membrane similarly to cell-penetrating peptides (CPPs) (Henriques,ST, et al. 2006). TLRs have been linked to both aseptic and septic loosening of prostheses due to their interaction with pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Takagi,M, et al. 2007, Tamaki,Y, et al. 2009).

hBD-3 has been shown to be involved in wound healing (Sorensen,OE, et al. 2003, Sorensen,OE, et al. 2006), as the secretion of hBD-3 and other antimicrobial peptides by human keratinocytes was found in sterile wounds and microbe-induced wounds. Additionally, hBD-3 has been discovered in the synovial membrane of osteoarthritis, psoriatic arthritis (Paulsen,F, et al. 2002) and rheumatoid arthritis (Bokarewa,MI, et al. 2003) patients without signs of infection, suggesting a role in sterile tissue inflammation states. As hBD-3 is secreted upon induction in inflammatory scenarios, it was not found in a healthy synovial membrane in contrast to the presence of hBD-1 (Paulsen,F, et al. 2002).

Recent interest has been towards the introduction of hBD-3 as an antimicrobial therapeutical tool and evaluation of its possible role in diagnostics. hBD-3 has been considered for diagnosis of PJI (Matsen Ko,L and Parvizi,J. 2016). Formalin-fixed and paraffin-embedded tissue sections were analyzed using indirect immunofluorescence and mean optical density. Samples from septic loosening showed the highest values of hBD-3, followed by aseptic loosening, the spacer group, and healthy controls (Liu,GD, et al. 2014). This indicates a local increase in the proportion and/or intensity of staining of hBD-3 immunoreactive cells around septically loosened implants. Gollwitzer et al. studied the presence of hBD-3 in synovial fluid in septic and aseptic loosening. Increased concentrations of hBD-3 were found in synovial fluid of septic loosening patients compared to aseptic loosening group (Gollwitzer,H, et al. 2013). As an antimicrobial agent, hBD-3 was more effective in comparison with vancomycin and clindamycin (Huang,Q, et al. 2012). hBD-3 showed antimicrobial and antibiofilm activity against two different MRSE and MRSA, as reported by Zhu et al. (Sutton,JM and Pritts,TA. 2014, Zhu,C, et al. 2013).

3. THE AIMS OF THE STUDIES

The aim of this thesis was to obtain more information of interaction between microbes and different biomaterials and the localization of hBD-3 in peri-implant tissues in septic joint implant loosening. The following questions were addressed:

-to test the bacterial adhesion inhibition ability of DLC compared to conventional biomaterials used in orthopedics in the presence of serum

-to compare DLC and two novel DLC polymer hybrid coatings to biomaterials traditionally used in orthopedics for their ability to inhibit *S.aureus* and *S.epidermidis* adhesion under dynamic flow conditions

-to compare DLC and two novel DLC polymer hybrid coatings to biomaterials traditionally used in orthopedics for their ability to resist *S.aureus* and *S.epidermidis* biofilm formation

-to identify the cellular sources of hBD-3 expression in septic joint implant loosening with peri-implant tissue samples

4. MATERIALS AND METHODS

This thesis consists of four studies related to PJI. Three of them (I, II and IIII) are focused on the evaluation of bacterial adhesion and biofilm formation on novel implant coating materials in comparison to commonly used implant materials. The fourth study (IV) evaluates the cellular sources of hBD-3 expression in peri-implant tissues of septic joint implant loosening patients.

Table 2. Summary of Materials and Methods

Study	Materials/Samples	Sample fabrication	Bacteria	Pre-treatment and sample characterization	Stainings	Testing methods
I	Patterned: Ta, Ti, Cr, DLC	UV-lithography, FPAD, DC sputtering	*S.aureus S-15981*	Pre-incubation in FCS	Acridine Orange	epifluorescence microscopy, quantification of bacterial adhesion
II	Ta, Ti, Cr, DLC, DLC-PDMS-h, DLC-PTFE-h	FPAD, DC sputtering	*S.aureus S-15981, S.epidermidis ATCC 35984*	Contact angle, sliding angle, surface roughness	Syto 9	CLSM, quantification of bacterial adhesion
III	Ta, Ti, Cr, DLC, DLC-PDMS-h, DLC-PTFE-h	FPAD, DC sputtering	*S.aureus S-15981, S.epidermidis ATCC 35984*	Pre-incubation in FCS, contact angle, SFE and its components, surface roughness, zeta potential	Calcofluor White, Live/Dead	epifluorescence microscopy, CLSM, quantification of biofilm coverage
IV	Peri-implant tissue samples of septic implant loosening	Paraffinization	Variable	Data collection, microbial cultures	IHC: hBD-3 IF:hBD-3, CD 68, MCT, CD 31, HSP 47	light microscopy, qualitative analysis of tissue samples

Ta : tantalum, Ti : titanium, Cr : chromium, DLC : diamond-like carbon, DLC-PDMS-h : diamond-like carbon polydimethylsiloxane hybrid, DLC-PTFE-h : diamond-like carbon polytetrafluoroethylene hybrid, UV-lithography : ultraviolet-lithography, FPAD : filtered pulsed arc discharge method, DC sputtering : direct-current sputtering, CLSM : confocal laser scanning microscopy, IHC : immunohistochemistry, IF : immunofluorescence, FCS: fetal calf serum.

4.1 Biomaterial processing

The biomaterial samples were prepared on Si wafers. UV-lithography was used for patterning (Study I), and the coatings were deposited using the direct-current (DC) sputtering technique for metals (tantalum, titanium, and chromium) and FPAD method for DLC and DLC polymer hybrid films (Studies I, II and III).

4.1.1 Silicon wafers (Studies I, II and III)

Silicon wafers (p-type, single crystal <100>) 101.6 mm in diameter and 0.68 mm thick (Okmetic, Vantaa, Finland) were used in the dynamic adhesion study and 101.6 mm in diameter and 0.5 mm thick (Okmetic, Vantaa, Finland) in the static adhesion assay. In biofilm study, 0.25 mm thick and 127 mm in diameter, silicon wafers (Si-Mat, Landsberg am Lech, Germany) were used. Before the deposition, silicon wafer surfaces were cleaned using argon sputter (SAM-7kV, Minsk, Belarus) in the vacuum. The initial vacuum chamber pressure was $8x10^{-4}$ Pa. The sputtering time was 10 min, and the voltage and current used were 5 kV and 30 mA, respectively. During the argon sputtering the vacuum chamber pressure was 0.01 Pa. The purity of argon was 99.999% (Instrument Argon 5.0, Oy AGA Ab, Espoo, Finland). After the argon sputtering, all deposition processes were continued without breaking the vacuum.

4.1.2 Ultraviolet-lithography (Study I)

UV-lithography was used in microfabrication in the first study to pattern parts of a thin film or the bulk of a substrate. A geometric pattern from a photomask was transferred to a light-sensitive chemical "photoresist" on the substrate. CleWin layout software (WieWeb software, Hengelo, The Netherlands) was used to design the photomasks, and the masks were fabricated using laser scanning technique by Mikcell Ltd. (Ii, Finland) on 101.6 mm glass plates with structured chrome layer.

For bacterial adhesion study, 10 mm x 10 mm sample chips were divided into four 4 mm x 4 mm areas. One of them was an unpatterned reference, and three were patterned with spots of different diameters (5, 25 or 125 μm). Regardless of the diameter of the spots, they coated 30.6% of the total surface area of the patterned quadrants. Sample chips were fabricated as follows: a) biomaterial spots on Si background and b) Si spots on biomaterial background.

20% hexamethyldisilazane (HMDS, Semiconductor Grade Puranal, Riedel-de Haen Laborchemikalien GmbH, Seelze, Germany) in xylene was spin coated on the cleaned and dried silicon wafers to establish adhesion between silicon wafers and photoresist. For this work, Epoxy-based negative photoresist SU-8 2003 (MicroChem, Newton, MA) was exposed to 365 nm UV light (Karl Suss MA45, Suss Microtec Inc., Waterbury Center, VT, USA) through the photomask. Unexposed SU-8 areas were removed using propylene glycol methyl ether acetate (PGMEA, Sigma-Aldrich, St.Louis, MO, USA) immersion, and sample chips were cleaned with isopropanol and deionized water in an ultrasonic bath (Madou,M. 1997, Voldman,J, et al. 1999).

4.1.3 Direct-current sputtering (Studies I, II and III)

Two physical vapor deposition (PVD) methods were used for deposition of thin films on silicon wafers: DC sputtering and FPAD method. DC sputtering is a physical method that was used to deposit thin films of metals on a substrate surface. High-energy ions are

produced in gaseous plasma and accelerated to bombard the solid sputtering metal target. The collision of high-energy Ar^+ ions with the metal target detaches atoms from it. They are then ejected and hit the substrate to be coated so that a thin film of the source material forms on the surface of the substrate.

More specifically, the Stiletto Series ST20 DC sputtering device (AJA International Inc., North Scituate, MA, USA) was used. To enable ignition of the plasma, the pressure was maintained in the range of 10^{-3} Pa. A negative target potential up to 400-500 V was applied in DC sputtering to accelerate the positively charged Ar+ ions to the metal target. The impacting Ar^+ ions removed material from the Ta, Ti or Cr target that was sputtered and deposited on the surface of the <100> silicon wafer (Okmetic Ltd, Vantaa, Finland). Deposition times for a 200±20 nm thick layer of tantalum, titanium, and chromium were approximately five minutes.

4.1.4 Filtered pulsed arc discharge (FPAD) (Studies I, II and III)

FPAD method was used to deposit thin films of DLC and modified FPAD method to deposit DLC-PTFE-h or DLC-PDMS-h coatings on the substrate surface (Figure 4.). In FPAD, a high-current electric arc strikes the graphite cathode vaporizing material (graphite particles, neutral atoms, and plasma ions) from it. This material is then accelerated using high voltage through a 90° curved solenoid to filter out large particles and neutral atoms before hitting the target material. In modified FPAD a graphite-polymer cathode (with the desired polymer) is used.

In this particular FPAD system high purity graphite cathode (99.9%, Carbone Lorraine, Paris, France), graphite-PTFE and graphite-PDMS cathodes (Irpola Oy, Turku, Finland) were used to deposit DLC, DLC-PTFE-h or DLC PDMS-h coatings, respectively, on <100> silicon wafer chips.

In the first step, an adhesion layer was deposited using high plasma energies. The main portion of the DLC/DLC-PDMS-h/DLC-PTFE-h film was deposited with a low energy deposition unit run at 500 V average voltage for 15 minutes. The thickness of DLC, DLC-PDMS-h, and DLC-PTFE-h films on the silicon wafers was 150 ± 20 nm in dynamic adhesion study (Study II) and 200 nm in biofilm study (Study III). In static adhesion assay (Study I) the DLC film thickness was 200 nm. The DLC coatings produced were hydrogen free and amorphous with high diamond sp^3-bond content (85%) (Anttila,A, et al. 1997).

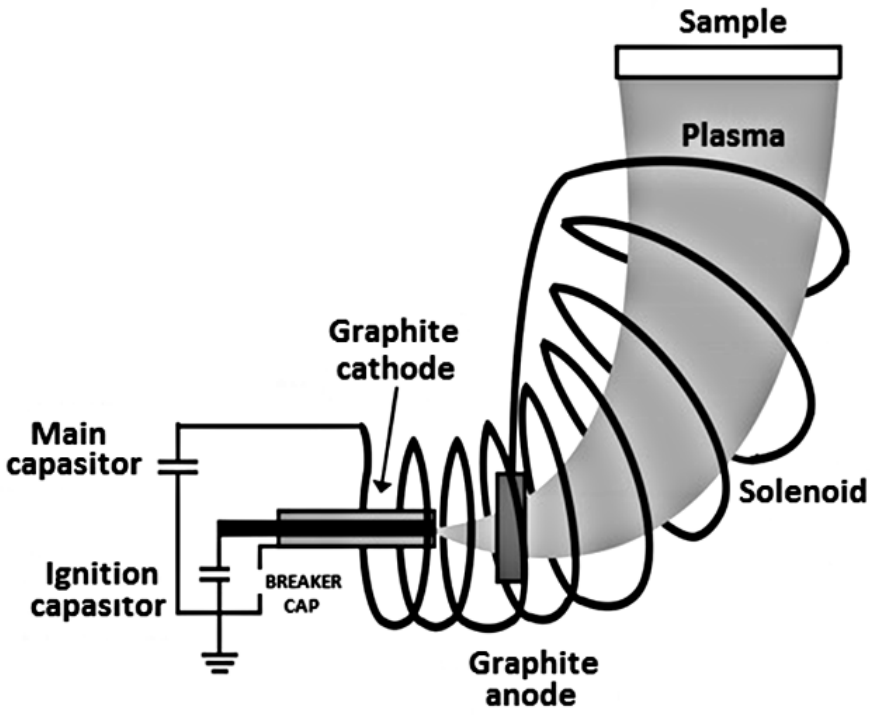


Figure 4. A schematic presentation of the filtered pulsed arc discharge (FPAD) method. Used with permission from: Soininen A. Studies of diamond-like carbon and diamond-like carbon polymer hybrid coatings deposited with filtered pulsed arc discharge method for biomedical applications. Unigrafia, Helsinki 2015, ISBN: 978-952-9657-78-0.

4.1.5 The processing of the coated silicon wafers and sample chips (Studies I, II and III)

After the coating deposition, silicon wafers were immersed in acetone or resist remover bath (mr-Rem 660, Micro Resist Technology GmbH, Berlin, Germany). In the study I, the final micropatterns were formed after the biomaterial coating deposited on the top of the resist was lifted off with the dissolved resist.

A device with a custom-made diamond knife was used to cut the silicon wafers into individual sample chips. The cutting apparatus was designed in such a way that it did not alter the surface chemistry or contaminate the sample chips during the cutting process. For static bacterial adhesion (Study I) and biofilm studies (Study III), silicon wafers were cut to 10 mm x 10 mm sample chips. For dynamic bacterial adhesion study (Study II), the wafers were cut to 15 mm x 30 mm sample chips (to perfectly fit the sample chambers of the dynamic testing machine).

Before the static adhesion assays (Study I), sample chips were rinsed with 70% ethanol, and gamma sterilized using 28 kGy irradiation at the VTT Technical Research

Centre of Finland. The samples were sterilized with ethanol (Ethanol Anhydricum Aa, Berner Oy, Helsinki, Finland) before dynamic microbial studies (Study II). Before the biofilm experiments (Study III), sample chips were cleaned in 70% ethanol for 45 minutes and rinsed with distilled water three times.

For the dynamic bacterial adhesion studies (Study II), biomaterial sample chips without serum pre-treatment were used. For the static bacterial adhesion study (Study I) and biofilm study (Study III), biomaterial sample chips were incubated in FCS (Perbio Science Belgium, Erembodegem, N.V Belgium) for 15 minutes at +37°C to mimic *in vivo* conditions. At the next step, the samples were washed three times in 10 mM phosphate buffered, 140 mM saline (PBS, pH 7.4). In some static bacterial adhesion experiments, patterned sample chips of four different biomaterials were manually fixed on the array plates with nail varnish.

4.2 Biomaterial characterization

4.2.1 Surface roughness (Studies II and III)

Average surface roughnesses (R_a) of the coatings were examined with a 2D stylus profiler (Sloan Dektak IIa, Veeco Instruments Inc., Santa Barbara, CA, USA) for each coating batch (Study II). A mask was placed on the sample for the coating period, and the thickness of the coating on the silicon wafer was measured from the step formed after removal of the mask.

For biofilm study (Study III), R_a was measured utilizing PSIA XE-100 (Park Systems Corp., Suwon, Korea) atomic force microscope (AFM) from randomly selected areas at three different locations on six sample chips per group. To scan areas of 2x2 µm over sample chips, an aluminum coated Acta-10 (ST Instruments B.V., LE Groot-Ammers, The Netherlands) silicon cantilever was used in a non-contact mode. A non-contact mode with the scan rate of 0.25 Hz was employed. The results were analyzed using the instrument analysis software (XIA).

4.2.2 Contact and sliding angle (Studies II and III)

Contact angle (θ_c) measurements were done using the static sessile drop method using 15-20 µl droplets of distilled water and/or diiodomethane. Contact angles were evaluated with a CCD video microscope (Study II) and an optical microscope SZ-PT Olympus equipped with a digital Olympus Camedia C-3030ZOOM camera (Olympus Corp., Tokyo, Japan) (Study III). Five sessile drops per sample chip were processed using GIMP image analysis software (www.gimp.org), which calculated both the left and right contact angles from the shape of the drop (Study III). The contact angles of the samples were also measured after incubation in FCS (Perbio Science N.N., Erembodegem, Belgium) for 15 minutes at +37ºC in the biofilm study (Study III). The sliding angles were measured with 20 µl distilled water droplets in dynamic adhesion study (Study II). The sliding angle is defined as the critical angle at which a droplet begins to slide down an inclined plane. The accuracy of a single contact or sliding angle measurement was ±0.5°.

4.2.3 Surface free energy (Study III)

The total SFE and its polar/dispersive components of the sample surfaces were obtained. The dispersive γ_S^D and polar γ_S^P components of the surface free energies of the biomaterials studied were calculated using Owens-Wendt theoretical model (Owens,DK and Wendt,RC. 1969),

$$(1 + \cos\theta)\gamma_L = 2\left((\gamma_S^D\gamma_L^D)^{\frac{1}{2}} + (\gamma_S^P\gamma_L^P)^{\frac{1}{2}}\right) \quad (3)$$

Subscripts *S* and *L* represent solid and liquid surfaces, respectively. *D* refers to the dispersive component and *P* to the polar component of the surface tension. Surface tension components of the test liquids were γ_L = 72.8 mJ/m^2, γ_L^D = 21.8 mJ/m^2 and γ_L^P = 51.0 mJ/m^2 for water; and γ_L = 50.8 mJ/m^2, γ_L^D = 50.8 mJ/m^2 and γ_L^P = 0.0 mJ/m^2 for diiodomethane (Azeredo,J, et al. 1997). Total surface free energy γ_S is the sum of its dispersive and polar components.

4.2.4 Zeta potential (Study III)

Zeta potentials of naive and/or fetal calf serum pre-treated sample chips were measured using an electrokinetic analyzer (SurPASS, Anton Paar GmbH, Graz, Austria). An electrolyte (0.001 M KCl, pH 7.4±0.5) circulated through the measuring cell. It was forced under pressure to flow through a small 100 µm gap between two identical sample chips, and a corresponding movement of the charges in the electrochemical double layer occurred causing the zeta potential. Streaming current was measured between two Ag/AgCl electrodes located on both sides of the sample chips. The zeta potential (ζ) was calculated from streaming current measurements according to Helmholtz-Smoluchowski equation (Lyklema,J. 1995):

$$\zeta = \frac{dI}{dp} \times \frac{\eta}{\varepsilon \times \varepsilon_0} \times \frac{L}{A} \quad (4)$$

$\frac{dI}{dp}$ denotes the slope of streaming current versus pressure, η is the electrolyte viscosity, ε_0 refers to the vacuum permittivity and ε is the dielectric constant of the electrolyte. *L* and *A* are the length and the cross-sectional area of the streaming channel, respectively. This method takes into consideration all conductivity effects, including surface conductance, which contribute to the zeta potential.

4.3 Patients and samples for evaluation of hBD-3 expression in septic loosening (Study IV)

The cellular origin and expression of hBD-3 were studied with peri-implant tissue samples obtained from septic joint implant loosening patients. The patient study group consisted of six males and six females, a total of 12 patients with mean age of 72 years and range 58-87 years. Samples were obtained during revision operations from active sites of the joints from the inner side of the pseudo capsule and/or synovial membrane-like

interface membrane. Septic hip implant loosening was confirmed in seven patients, and five patients had septic knee implant loosening (Table 3.).

The samples were placed in 10% neutral formalin after removal, and after 24 hours they were dehydrated in an increasing ethanol series, cleared in xylene, embedded in paraffin and finally stored at room temperature. The informed consent form was signed by all the patients. The local Institutional Review Board of Orthopedic Hospital and the Slovenian Medical Committee (No 40/06/11) approved the study entitled "Evaluation of different methods for joint prosthesis infection determinations". In the diagnostic workup, the preliminary/working diagnosis was set to septic implant loosening after appropriate clinical examination, laboratory testing, and radiological imaging. Septic joint implant loosening was confirmed as the diagnosis by intraoperative bacterial cultures. All patients had a metal-on-ultra high molecular weight polyethylene (MoP) articulating pairs in implants.

4.4 Bacterial cultures

4.4.1 Static cultures (Study I)

A biofilm producing strain of *S. aureus* S-15981, kindly provided by Dr. Lasa and isolated at the Microbiology Department of the University Clinics of Navarra, Spain, was used in the static bacterial adhesion study (Valle,J, et al. 2003). Cultures were done on blood-agar plates. At first, twenty colony forming units (CFU) were suspended in ten mL of Tryptic Soy Broth (TSB, Bacto Tryptic Soy Broth, Becton & Dickinson and Company, Sparks, NJ, USA) and cultured for 24 hours at +37°C. Refreshing of the bacterial suspension was done by diluting one mL of the bacterial suspension with nine mL of TSB and culturing for 18 hours. At the next step, bacteria were suspended and diluted in PBS to final concentration $5x10^8$ CFU/mL according to the McFarland standard (Chapin,KC and Lauerdale,T. 2007). This was controlled by dilution plating on blood agar (Trypticase Soy Agar, BBL 211047, Becton & Dickson and Company, Sparks, NJ, USA and Mueller-Hinton Agar (BBL 212257, Becton & Dickinson and Company, Sparks, NJ, USA, supplemented with 5% horse blood). Two mL of $5x10^8$ CFU/mL bacterial suspension was pipetted to each well containing a biomaterial sample chip. Eight-well culture plates were used. In some experiments, 15 mL of the bacterial suspension was pipetted on the biomaterial array plates (custom-made glass plates were used to facilitate sample chip handling, and four different biomaterials sample chips were attached to bottom of these plates with nail varnish, and the whole constructs were placed to ten cm Petri dishes). The incubation time for cultures at +37°C was 90 minutes, to allow adhesion of bacteria. Finally, individual sample chips or sample chips on array plates were washed three times with distilled water to remove non-adherent bacteria. All sample chips or arrays were studied in triplicate.

Table 3. The characteristics of septic loosening patients and associated microbiological pathogens. The column about the type of pathogens provides the number of positive cultures from the total number of cultures taken. Modified from Levon *et al*. 2015 (Levon,J, et al. 2015).

Sample	Gender	Age	Location of the prosthesis	Pathogen	Prosthesis survival time (months)
1	male	71	knee	Anaerobic Gram+ cocci (4/6), anaerobic Gram + bacilli (4/6), *Finegoldia magna* (2/6)	10
2	male	61	knee	*Candida parapsilosis* (6/6), *S. epidermidis* (1/6)	9
3	male	85	knee	*S. aureus* (1/7), synovial fluid negative, sonication positive	25
4	female	76	knee	*S. aureus* (2/6)	3
5	male	72	knee	*Enterobacter cloacae, S. sciuri, Enterococcus faecalis, Corynebacterium striatum* (6/6)	1
6	male	65	hip	*Enterococcus faecalis* (5/6), *S. epidermidis* (5/6)	0.7
7	female	69	hip	*Kocuria* species (3/4 sonicates), *Propionibacterium acnes* (3/6)	100
8	female	59	hip	*S. aureus* (6/6)	68
9	female	87	hip	*S. epidermidis* (3/5)	51
10	female	78	hip	*S. aureus* (6/6)	3
11	female	75	hip	negative (0/5), synovial fluid *Corynebacterium striatum*	159
12	male	57	hip	Group G-beta haemolytic *streptococci, S. epidermidis, Corynebacterium striatum* (5/5)	0.6

4.4.2 Dynamic cultures (Study II)

S. aureus S-15981 and *S. epidermidis* ATCC 35984 strains were selected for dynamic bacterial adhesion experiments and transported to the University of Patras, Greece, where

the experiments were done. The biofilm forming reference strain *S. epidermidis* ATCC 35984 was originally isolated in Tennessee, USA.

The bacteria were stored at -70ºC, in a solution containing 70% TSB (Difco Laboratories, Detroit, USA) and 30% diluted glycerol (glycerol/water: 1/1). Ten µL aliquots of the frozen bacterial suspension of each strain were cultured on blood agar plates overnight at +37°C. The plates were subsequently stored at +4°C. Two to three bacterial colonies from the refrigerator stored blood agar plates were incubated in five mL TSB for 18 hours at +37°C in a rotatory shaker at 120 rounds per minute to produce stationary phase bacterial cells. Bacteria were harvested by centrifugation at a centrifugal force of 2683 x g at +4°C for ten minutes and washed twice with a phosphate buffered saline buffer, pH 7.4, with an ionic strength of 180 mM, consisting of 100 mM phosphate buffered saline supplemented with 80 mM NaCl. Finally, the bacteria were resuspended in the same buffer to 1.5×10^8 CFU/mL concentration according to the McFarland standard (BioMerieux, SA Lyon, France).

This was done using optical density measurements of the bacterial suspensions at 550 nm with a spectrophotometer (Techne, Cambridge, UK).

The parallel plate flow chamber (PPFC) was used to study bacterial adhesion under dynamic flow conditions. The 30 mm × 15 mm × 1 mm biomaterial sample chip was sandwiched between two plexiglass plates in such a way that a 30 mm × 15 mm × 0.35 mm parallel plate flow chamber was formed. Four syringes were placed in an automated syringe pump and connected to four different flow chambers. The pump cycled the pistons back and forth every 60 seconds continuously, at which time 3.7 mL were displaced. This process cycled for 120 minutes. All experiments were carried out at +37°C, and the shear rate was adjusted to 200 s^{-1}.

The shear rate γ was calculated by the following formula:

$$\gamma = \frac{6Q}{Wh^2} \tag{5}$$

Where Q is the flow rate, W (width of the chamber) = 15 mm and h (height of the chamber) = 0.35 mm. Each experiment was performed in three replicates.

4.4.3 Biofilm cultures (Study III)

S. aureus S-15981 and *S. epidermidis* ATCC 35984 were grown on blood agar plates. Twenty CFU were suspended into ten mL of TSB (Bacto Tryptic Soy Broth, Becton & Dickinson and Company, Sparks, NJ, USA) and cultured for 24 hours at +37°C. The bacterial suspension was refreshed for the experiments by diluting one mL of the bacterial suspension with nine mL of TSB, followed by culture for 18 hours. After this, the bacteria were diluted and resuspended in TSB to obtain a concentration of $5x10^8$ CFU/mL according to McFarland standard (Chapin,KC and Lauerdale,T. 2007). The concentration was controlled by dilution plating on blood agar (Trypticase Soy Agar, BBL 211047, Becton & Dickson and Company, Sparks, NJ, USA and Mueller-Hinton Agar, BBL 212257, Becton & Dickinson and Company, Sparks, NJ, USA, supplemented with 5%

horse blood). Two milliliters of $5x10^8$ CFU/mL of bacterial suspension was pipetted into each well of a 12-well plate containing biomaterial sample chips and incubated at +37°C for 16 hours. The selected 16 hours incubation time was based on pilot experiments (data not shown). After 16 hours of biofilm forming incubation, the sample chips were washed three times with 0.9% NaCl before analysis of the biofilm-coated area on the biomaterial sample chips. All sample chips were run as duplicates, and the experiments were repeated three times with both staphylococcal bacterial strains.

For biofilm viability studies using Live/Dead Baclight staining, bacteria were cultured overnight at +37°C in TSB (Bacto Tryptic Soy Broth Becton, Dickinson, and Company Sparks USA). The concentration was adjusted to $5x10^8$ CFU/mL in TSB. Two milliliters of bacterial suspension per well was added to the eight-well culture plates containing FCS (Perbio Science Belgium N.V Belgium) pre-treated biomaterial sample chips on the bottom of the wells for 24-hour incubation at +37°C. After this, the bacterial suspension was refreshed by aspiration and replaced with fresh TSB. After a total 48 hours of incubation, the sample chips were washed three times with distilled water to rinse away free bacteria and buffer before Live/Dead Baclight staining.

4.5 Stainings

4.5.1 Acridine Orange staining (Study I)

Acridine Orange 3R nucleic acid selective fluorochromic cationic stain (Chroma-Gesellschaft Schmid GmbH, Köngen, Germany) was used to visualize adherent *S. aureus* cells on patterned sample chips as such or array plates. The sample chips were incubated for two minutes in 1:10 000 (w/v) Acridine Orange 3R in 0.2 M acetate buffer, pH 3.8. Acridine Orange staining solution was stored in the dark at room temperature. After Acridine Orange 3R staining the sample chips were rinsed with tap water before epifluorescence microscopy and photographing for morphometric analysis.

4.5.2 Syto 9 staining (Study II)

After dynamic flow adhesion cultures, all sample chips still mounted in the flow chambers were rinsed with eight milliliters of buffer solution to remove non-adherent or loosely adherent bacteria. Biomaterial sample chips placed in chambers were fixed in freshly prepared 3% formaldehyde for 30 minutes at +37°C. Formaldehyde was made by adding paraformaldehyde (Sigma, Missouri, USA) in 100 mM sodium phosphate buffer at +60°C, followed by drop-wise addition of NaOH until the solution was clear. After fixation, the sample chips were stained in one mL (in each chamber) of a freshly prepared solution containing two µL SYTO 9 (Molecular Probes Europe BV, Leiden, The Netherlands) per one mL of deionized water for 30 minutes at room temperature, in the dark. The distribution of adherent bacteria was visualized with Syto 9. Syto 9 penetrates the staphylococcal cell membrane and is a fluorescent DNA-binding stain. After staining the sample chips were rinsed three times with distilled water, the chambers were

dismounted, and the sample chips placed on object slides and mounted in Gel Mount (Sigma, Missouri, USA), an aqueous mounting medium. Finally, the sample chips were covered with a coverslip before confocal laser scanning microscopy.

4.5.3 Calcofluor White staining (Study III)

Fluka 18909 Calcofluor white stain (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was pipetted on biomaterial chips containing *S. epidermidis* and *S. aureus* biofilms for two minutes. Calcofluor white is a fluorochrome stain, and it interacts with the polysaccharide components of the biofilm. The biofilms on the surfaces of the biomaterial sample chips were washed with 0.9% NaCl three times for five minutes after Calcofluor white staining before epifluorescence microscopy.

4.5.4 Live/Dead viability staining (Study III)

Live/Dead Baclight viability staining was used to observe biofilm on biomaterial sample chips. With the LIVE/DEAD Baclight Bacterial viability kit (L7012, Invitrogen Molecular Probes Eugene, Oregon, USA), a stock dilution was made. In this, equal volumes of Syto 9 (nucleic acid stain, excitation 490 nm/emission 522 nm) and Propidium Iodide (nucleic acid stain, excitation 490 nm /emission 635 nm) were combined. Viable cells are stained green by lipophilic Syto 9, while Propidium Iodide, which penetrates only non-viable cells with damaged cell membranes, stains dead cells red. For every three µL of such solution, one mL of distilled water was added. The stock dilution was mixed, and the sample chips were incubated with it for 15 minutes in the dark at room temperature. Distilled water was used to wash the sample chips three times after staining before confocal laser scanning microscopy.

4.5.5 hBD-3 immunohistochemical staining (Study IV)

For immunohistochemical staining of hBD-3 in peri-implant tissues of septic implant loosening, four µm thick sections were used. The first section was stained with eosin and hematoxylin, after which the following sections were used for immunostaining. A pilot titration experiment was run to find the optimal antibody concentrations. In this experiment, a serial dilution of the primary antibodies was used to stain five consecutive deparaffinized tissue sections. An entirely automatic staining of tissue sections was performed with Bond Polymer Refine (DS9800) horseradish peroxidase (HRP) labeled polymer method after placement of the sections in a Leica-Bond-Max immunostainer (Leica Microsystems GmbH, Wetzlar, Germany). A citrate buffer solution was used for antigen retrieval for 20 minutes. According to the staining protocol, the sections were first incubated for 30 minutes in 0.25 µg/mL affinity-purified rabbit anti-human hBD-3 IgG (Novus Biologicals, Littleton, CO, USA). After that, the slides were incubated for eight minutes in Post Primary and eight minutes in the anti-rabbit Poly-HRP-IgG polymer. Ten minutes of incubation in 0.023% 3,3´diaminobenzidine tetrahydrochloride Mix DAB Refine was done and finally five minutes of incubation in hematoxylin. A washing buffer supplied by the company was used to wash the slides between the steps. Slides were

dehydrated and mounted in Mountex (HistoLab, Gothenburg, Sweden). As positive and negative staining controls, tissue sections from periodontitis lesions and non-immune rabbit IgG (Jackson ImmunoResearch 011-000-003, Suffolk, UK) at the same concentration as and instead of the primary rabbit anti-human BD-3 IgG, respectively, were used.

4.5.6 Double immunofluorescence staining (Study IV)

For immunofluorescence staining, antigen retrieval was done in citrate buffer pH 6.0 using a microwave oven for ten minutes at +98°C (MicroMED T/T Mega Histoprocessing Labstation; Milestone Srl, Sorisole, Italy) after deparaffinization of the tissue sections. After a wash with PBS three times for five minutes, the slides were permeabilized with Triton X-100 for ten minutes at room temperature (RT). Slides were washed with PBS three times for five minutes and incubated with 10% normal goat serum for one hour at RT. After the serum blotting, the slides were incubated with four µg/mL affinity-purified rabbit anti-human hBD-3 IgG together with one of the selected antibodies. 0.5 µg/mL mouse anti-human CD68 IgG1 (DAKO, Glostrup, Denmark), 0.1 µg/mL mouse anti-human mast cell tryptase IgG1 (MST, AbD Serotec, Oxford, UK), 22.5 µg/mL mouse anti-human CD31 IgG1 (DAKO, Glostrup, Denmark) or 10 µg/mL mouse anti-human HSP47 IgG2b (StressGen Biotechnologies Corp., Victoria, BC, Canada) was used for overnight at +4°C. This was followed by washes in PBS three times for five minutes and incubation in 5 µg/mL of secondary Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (both from Alexa Fluor, Molecular Probes, Leiden, The Netherlands) for one hour at RT. Then the slides were washed in PBS three times for five minutes and subsequently incubated in 4'6-diamidino-2-phenylindole (DAPI) for ten minutes at RT. At the next step, the slides were washed in PBS two times for five minutes and in distilled water for five minutes, followed by mounting in Vectashield® mounting medium (Vector Laboratories, Burlingame, CA). Non-immune rabbit IgG, mouse IgG1, and mouse IgG2b were used for negative staining controls at the same concentrations as and instead of the primary antibodies.

4.6 Microscopy

4.6.1 Epifluoscence microscopy (Studies I, III and IV)

Acridine Orange 3R positive bacteria were visualized under an epifluorescence microscope (Olympus AX70, Hamburg, Germany) with 460-495 nm excitation and 510 nm emission wavelength U-MWIB3 FITC filters (Study I). They were photographed using PCO Sensicam (PCO AG, Kelheim, Germany) digital camera attached to a computer for subsequent morphometric analysis.

Calcofluor white stained biofilms were visualized under an epifluorescence microscope (Olympus Optical Co, Tokyo, Japan) using DAPI-filter (Study III). The biofilms were photographed with a digital camera (Olympus Optical Co, Tokyo, Japan) coupled to an Analysis software program (Soft Imaging System GmbH, Münster,

Germany) for subsequent morphometric analysis. 19 images of each sample chip were taken from similar locations to represent biofilm coverage on the sample surface.

The immunofluorescence stained tissue sections were analyzed and photographed using Leica DM6000 microscope and photographed using DEC420 color camera (Leica Microsystems, Buffalo Grove, IL, USA) (Study IV).

4.6.2 Confocal Laser Scanning Microscopy (Studies II and III)

Nikon TE2000-U Confocal Laser Scanning Microscopy (CLSM) system was used (Nikon, The Netherlands) to evaluate bacterial adhesion to the sample chips and to enumerate the adherent bacteria (Study II). In the system, a laser scanning module was mounted on an inverted microscope. The argon laser was utilized, and the images were recorded at an excitation wavelength of 488 nm and an emission wavelength of 500 nm. Since the adherent bacteria only formed monolayers, a thin section was scanned and saved as a bitmap image. Six fields of 100 μm^2 area were chosen randomly, and six non-overlapping images were taken from each sample chip to eliminate the possible effect of a slightly uneven distribution of bacteria (Study II).

Confocal microscopy was done to visualize the morphology of the bacterial biofilms and live and dead bacteria using a Leica TCS SP5 (Leica Microsystems, Mannheim Germany) microscope with a dipping water immersion objective HCX APO L 63x/0.9 W (Study III).

4.6.3 Light microscopy (Study IV)

For localization of hBD-3 in peri-implant tissues in septic implant loosening, the analysis and photography of immunohistochemically stained slides were done by using a Leitz Diaplan microscope and five megapixels Leica DFC420 digital camera (Leica Microsystems, Wetzlar, Germany). Several factors were considered in the analysis of the stained sections. The origin of the sample (hip, knee); the type of the pathogen (Staphylococci + or -); the survival time of the implant (the time from the primary operation to the revision operation) and the gender (female, male). The analysis was done separately for the hip and knee joints.

4.7 Quantification of bacterial adhesion and biofilm formation

4.7.1 Static bacterial adhesion (Study I)

To produce images showing areas of equal size of the different surfaces for analysis of bacterial adhesion, a masking layer with four circles of ~91 μm diameter that presented the image below was created in Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA, USA). These images were processed by setting the background black in each picture. Individual images were opened in ImageJ 1.37c (National Institute of Health, Bethesda, MD, USA) and the bacterial coverage area in each image was calculated using the nucleus-counter function of the WCIF plug-in (Abramoff,M, et al. 2004, Rasband,W.

2007, Rasband,W. 2007). All results represent triplicates, shown using mean ± standard deviation of the mean (SD).

4.7.2 Dynamic bacterial adhesion (Study II)

Digital image analysis of the CLSM pictures and the quantification of adherent bacteria were done using the software developed at the University Medical Centre Groningen, the University of Groningen in The Netherlands. The software is based on the methods described earlier (Meinders,JM, et al. 1992).

SPSS statistical software, version 14.0 (SPSS, Chicago, IL) was used statistically to examine the effect of the biomaterial properties on bacterial adhesion. The Shapiro-Wilk (W) test was utilized to analyze the normal distribution of the variables. All variables were normally distributed because W was close to one for all of them. Subsequently, one-way analysis of variance (ANOVA), and Post-hoc comparisons of all possible combinations of group means were done utilizing the Scheffe significant difference test. $P<0.05$ was considered to be significant.

4.7.3 Biofilm coverage (Study III)

All 19 images were analyzed and segmented using Otsu's thresholding method. The proportion of bacterial biofilm coverage on the material surfaces was calculated using MATLAB (The MathWorks Inc., Natick, MA, USA). One-way ANOVA (SPSS 16.0 software) variance analysis and Tukey Post-Hoc Tests were applied to determine the statistical significance of the differences between groups. $P<0.05$ was considered as significant.

5. RESULTS

5.1 Static bacterial adhesion (Study I)

In static bacterial adhesion tests 1) homogenous, non-patterned biomaterial surfaces and 2) samples containing the same biomaterials as spots on silicon background (Figure 5 A and B) or, *vice versa*, providing silicon spots on biomaterial background (Figure 5 C and D), were compared against each other. The conventional implant materials Ta, Ti and Cr and DLC, were studied for their ability to inhibit adhesion of *S. aureus* bacteria. Tantalum and titanium surfaces acted as good adhesion substrates for *S. aureus*, whereas chromium and DLC inhibited bacterial adhesion with almost no bacterial cells adhering to them (Figure 5.).

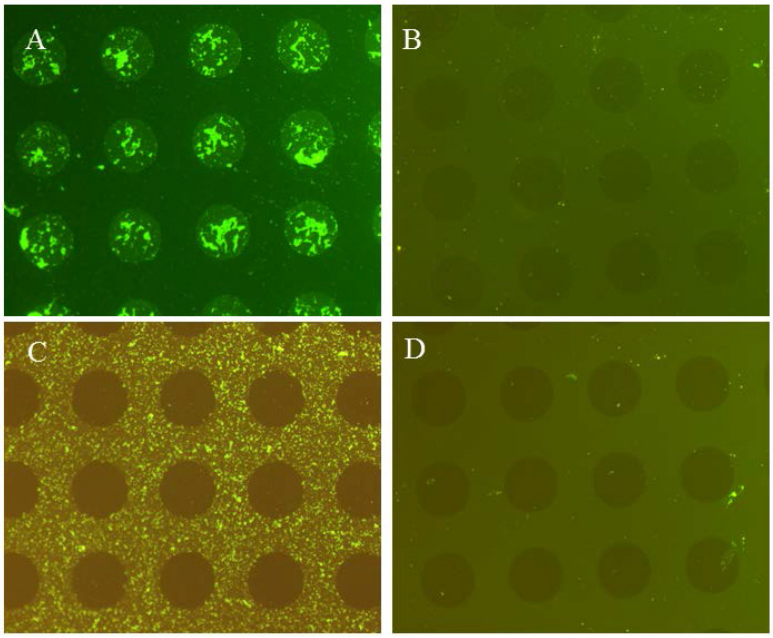
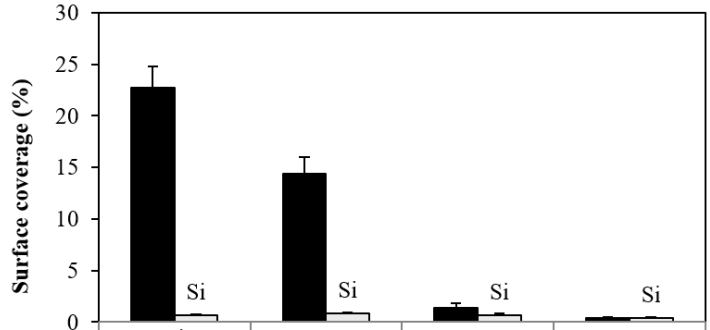


Figure 5. *S.aureus* adhesion to microtextured material samples. The first column shows 125 µm titanium biomaterial circles on silicon background (A) and 125 µm silicon circles on titanium biomaterial background (C). In the second column, 125 µm DLC biomaterial circles are seen on silicon background (B) and 125 µm silicon circles on DLC biomaterial background (D). Acridine orange staining, original magnification x 10.

When the bacterial resistance of different biomaterials on silicon background or silicon patterns on biomaterial background were compared, the results were clear. *S. aureus* cells grew firmly only on tantalum and titanium patterns or on tantalum and titanium background, but not on the silicon patterns or silicon background, which were almost free of bacteria. *S.aureus* adhesion was low on DLC and chromium not dependent on whether they formed patterned spots or background. In conclusion, DLC, chromium, and silicon

did bind considerably fewer bacteria in all experimental settings when compared to Ti and Ta.

The percentage area of the biomaterial covered by adherent bacteria was calculated (Figure 6.), and this showed clearly the preference of *S. aureus* bacteria to tantalum and titanium materials over DLC and chromium.



	Ti	Ta	Cr	DLC
■Binding to biomaterial circles*	22,69	14,34	1,41	0,38
□Binding to silicon background**	0,68	0,84	0,66	0,40

Figure 6. Static *S.aureus* adhesion. Adhesion of bacteria to biomaterials (black bars) and silicon background (white bars) used as an internal standard in 90-minute long adhesion experiment under static conditions. Circular spots with 125 µm diameters of four different biomaterials were tested against silicon background and each other. Values are the mean ± S.D. DLC = diamond-like carbon, Ta = tantalum, Ti = titanium, Cr = chromium.
*Expressed as the percentage of the circular biomaterial area covered by the adherent bacteria.
**Expressed as the percentage of the silicon background area covered by the adherent bacteria.

The effect of the size of the patterned spots on bacterial adhesion was evaluated. Different sized patterns were compared to each other and homogenous biomaterial area. Each patterned sample chip contained four different biomaterial areas (quadrants), three of which had micro-texturized spots whereas one quadrant area was coated homogeneously. 125 µm, 25 µm or 5 µm patterned circular spots were compared to the material surface without any texture. As the total amount of adherent bacteria on chromium and DLC were minimal, the effect of the size of the pattern (texture) on the adherence of *S. aureus* could not be evaluated further using these biomaterials. With Ta and Ti, some conclusions could be made. On the sample areas with patterned large 125 µm and intermediate 25 µm spots bacteria adhered either to a) metal-coated circular areas, but were not present on the silicon background or to b) metal background if metal was located in the background interrupted by regularly spaced circular silicon spots. In contrast, with the small 5 µm titanium and tantalum spots, bacteria did not seem so strictly to respect the borders of the circular features against the silicon background (and *vice versa*). *S.aureus* bacteria adhered both to the metal biomaterial and silicon, on the area presenting the small 5 µm features. Finally, on the plain biomaterial surfaces, bacteria

were organized to relatively homogenous bacterial coating and formed rather uniform mats.

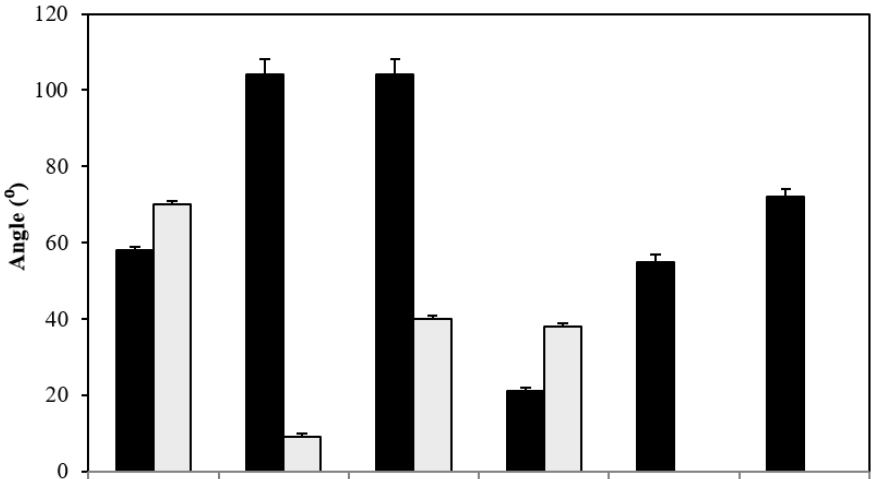
5.2 Dynamic bacterial adhesion (Study II)

S. aureus and S. epidermidis adhesion to biomaterial surfaces in dynamic flow conditions was enumerated. The number of bacteria on different materials, counted using image analysis software are shown in Table 5. *S. aureus* adhesion to DLC-PTFE-h and to tantalum was significantly ($P<0.05$) lower than to DLC-PDMS-h ($0.671\pm0.109 \times 10^7/cm^2$ and $0.751\pm0.223 \times 10^7/cm^2$ *vs.* $1.055\pm0.151 \times 10^7/cm^2$, respectively) (Table 4.). No other significant differences were detected in performed tests. These results also showed that *S. epidermidis* adhered equally to all materials tested ($P<0.05$).

Table 4. The numbers of *S. aureus* S-15981 and *S. epidermidis* ATCC 35984 bacteria adhered on DLC, DLC-PDMS-h, DLC-PTFE-h, Ta, Ti and Cr surfaces after 120 minutes of dynamic bacterial adhesion incubation expressed in units $1/cm^2$. Values are the mean ± S.D. DLC = diamond-like carbon, DLC-PDMS-h = DLC polydimethylsiloxane hybrid, DLC-PTFE-h = DLC polytetrafluoroethylene hybrid, Ta = tantalum, Ti = titanium, Cr = chromium.

Dynamic staphylococcal adhesion		
Biomaterial	*S. aureus*	*S.epidermidis*
DLC	$0.757 \pm 0.308 \times 10^7$	$0.448 \pm 0.123 \times 10^7$
DLC-PDMS-h	$1.055 \pm 0.151 \times 10^7$	$0.299 \pm 0.165 \times 10^7$
DLC-PTFE-h	$0.671 \pm 0.109 \times 10^7$	$0.413 \pm 0.189 \times 10^7$
Ta	$0.751 \pm 0.223 \times 10^7$	$0.354 \pm 0.015 \times 10^7$
Ti	$0.859 \pm 0.384 \times 10^7$	$0.402 \pm 0.107 \times 10^7$
Cr	$0.951 \pm 0.283 \times 10^7$	$0.475 \pm 0.080 \times 10^7$

The surface roughnesses, contact and sliding angles of the biomaterial samples were analyzed to evaluate their effects on bacterial adhesion. The highest contact angle values were measured for DLC-PDMS-h and DLC-PTFE-h, which were 104±4° for both; and the lowest sliding angle for DLC-PDMS-h, which was 9±1° (Figure 7.). The roughness values were 3±2 nm for DLC, DLC-PDMS-h, DLC-PTFE-h samples and 17±3 nm, 19±3 nm and 25±3 nm for tantalum, titanium, and chromium samples, respectively.



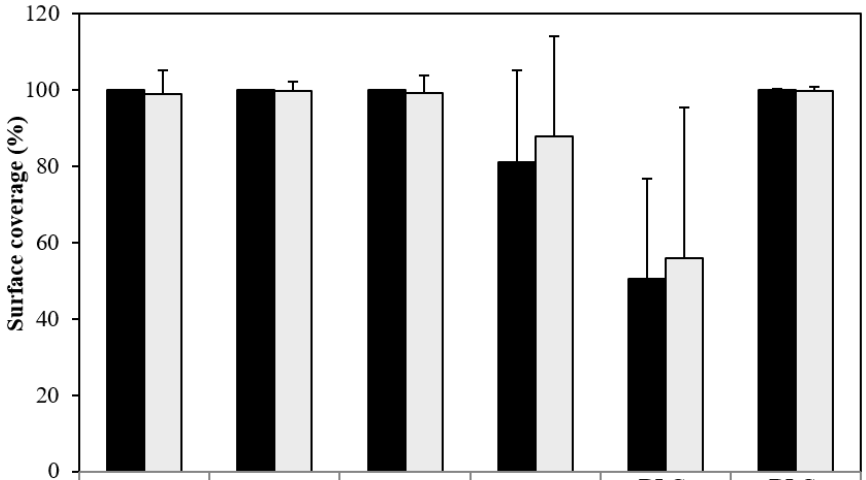
	DLC	DLC-PDMS-h	DLC-PTFE-h	Ta	Ti*	Cr*
■ Contact angle	58	104	104	21	55	72
□ Sliding angle	70	9	40	38		

Figure 7. Contact angle and sliding angle values of the biomaterials used in dynamic adhesion experiments. *denotes that droplet did not start to slide even at 90°. Values are the mean ± S.D. DLC = diamond-like carbon, DLC-PDMS-h = DLC polydimethylsiloxane hybrid, DLC-PTFE-h = DLC polytetrafluoroethylene hybrid, Ta = tantalum, Ti = titanium, Cr = chromium.

5.3 Biofilm formation (Study III)

5.3.1 Biofilm coverage

The conventional biomaterials Ti, Ta, and Cr, were almost entirely (close to 100%) covered by staphylococcal biofilm after 16 hours of biofilm incubation. DLC inhibited biofilm formation clearly better than metals and DLC-PDMS-h. 87.71±26.4% of the DLC surface area was covered by *S. epidermidis* biofilm and 81.05±24.1% by *S. aureus* biofilm (Figure 8.). DLC-PTFE-h was the best in the anti-biofilm ranking, with only 55.84±39.4% *S. epidermidis* and 50.52±26.3% *S. aureus* biofilm surface coverage, $P<0.001$ for both (Figure 8.). The anti-soiling and non-sticky DLC-PDMS-h did not differ in its biofilm formation inhibition from the conventional implant materials (Figure 8.).



	Ti	Ta	Cr	DLC	DLC-PTFE-h	DLC-PDMS-h
■ S.aureus	100	99,99	99,99	81,05	50,52	99,92
□ S.epidermidis	98,95	99,6	99,11	87,71	55,84	99,75

Figure 8. Biofilm surface coverage. The area covered by the biofilm (% of the total sample surface area) after 16 hours of *S.aureus* and *S.epidermidis* biofilm incubation. Values are the mean ± S.D. DLC and DLC-PTFE-h resisted significantly better biofilm formation compared to other materials (P<0.001)DLC = diamond-like carbon, DLC-PDMS-h = DLC polydimethylsiloxane hybrid, DLC-PTFE-h = DLC polytetrafluoroethylene hybrid, Ta = tantalum, Ti = titanium, Cr = chromium.

5.3.2 Surface roughness, contact angle, and surface free energy

The average surface roughness (R_a) values ranged from 0.6 nm to 2.0 nm (Table 5.). According to these measurements, all coatings were very smooth ($R_a \leq 2$ nm), similar to the mirror-finish surfaces of medical implants.

The contact angles and the total surface free energies, as well as their dispersive and polar components for all materials studied, are seen in Table 5. DLC-PTFE-h and DLC-PDMS-h had the highest water contact angles before serum incubation, 106° and 101.7°, respectively (Table 5.). They were followed by tantalum, DLC, titanium and chromium within the narrow 64.1^0-71.2° contact angle range. Serum protein pre-treatment caused a significant (P<0.001) drop in the water contact angles of all studied materials (Table 5.), this decrease being greatest (38%) for DLC-PTFE-h.

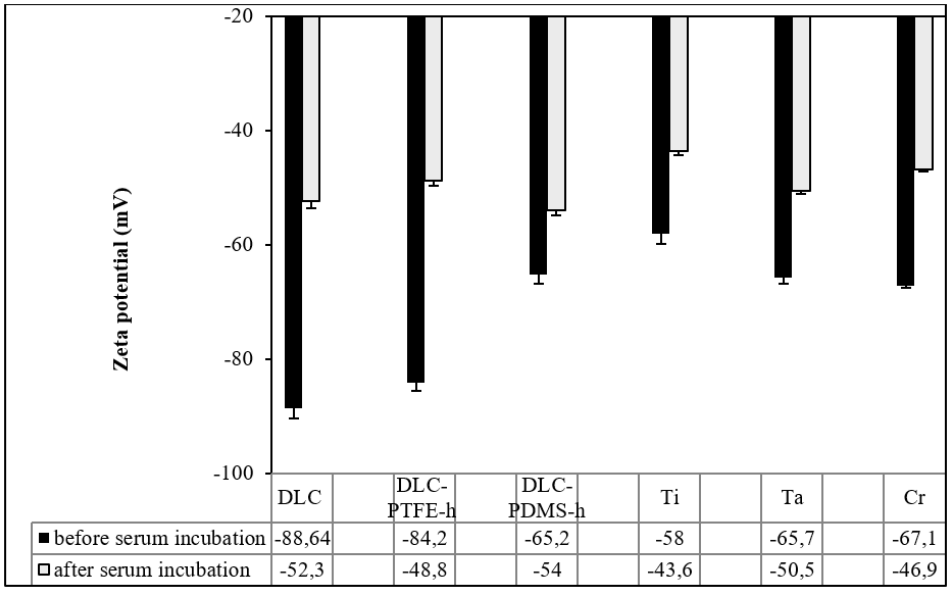
Table 5. Average surface roughnesses (R_a), water contact angles (θ^{water}) and surface free energy components for the surfaces of the tested materials. Water contact angles were measured both before and after serum incubation. DLC = diamond-like carbon, DLC-PDMS-h = DLC polydimethylsiloxane hybrid, DLC-PTFE-h = DLC polytetrafluoroethylene hybrid.

Measured biomaterial properties						
Materials	Roughness R_a (nm)	Contact angle θ^{water} (°)		Surface free energy components (mJ/m^2)		
		Before serum incubation	After serum incubation	γ_S^D	γ_S^P	γ_S
DLC	0.6±0.1*	67.4±2*	55.7±3.2*	36.7±0.7*	11.6±0.8*	48.3±1.0*
DLC-PTFE-h	2.0±0.1^{a}	106.0±1.1^{a}	65.9±5.1^{a}	23.7±0.6^{a}	0.8±0.2^{a}	24.5±0.5^{a}
DLC-PDMS-h	1.2±0.1^{a,*}	101.7±1.2 ^{a,*}	81.1±2.1^{a,*}	22.1±0.6^{a,*}	0.4±0.1^{a}	22.5±0.6^{a,*}
Titanium	1.8±0.2^{a,*}	67.6±2.0*	48.6±3.0*	34.9±0.7*	11.4±0.8*	46.3±0.3*
Tantalum	1.5±0.3^{a,*}	64.1±1.6^{a,*}	53.9±3.8^{a,*}	36.1±0.6^{a,*}	12.3±0.8^{a,*}	48.4±0.9^{a,*}
Chromium	0.8±0.1*	71.2±1.1^{a,*}	48.0±4.0^{a,*}	36.1±0.7*	9.1±1.0^{a,*}	45.2±0.7^{a,*}

Values are the mean ± S.D. , ^{a} $p < 0.05$ versus DLC , * $p < 0.05$ versus DLC-PTFE

5.3.3 Zeta potential

The average zeta potential values were negative for all the biomaterials studied (Figure 9.). Before serum protein pre-treatment, DLC and DLC-PTFE-h had the lowest zeta potentials, -88.4 mV and -84.2 mV ($p<0.001$ for both compared to the other materials), respectively. Cr, Ta, DLC-PDMS-h followed within a narrow range from -67.1 to -65.2 mV. Ti had clearly the highest value (-58.0 mV, $P<0.001$). Serum protein pre-treatment caused a significant ($P<0.001$) increase in the zeta potentials of all studied materials (Figure 9.). This increase was largest for DLC (41%) and DLC-PTFE-h (42%), but only 17% for DLC-PDMS-h.



	DLC	DLC-PTFE-h	DLC-PDMS-h	Ti	Ta	Cr
■ before serum incubation	-88,64	-84,2	-65,2	-58	-65,7	-67,1
□ after serum incubation	-52,3	-48,8	-54	-43,6	-50,5	-46,9

Figure 9. The zeta-potentials (ζ) of the tested materials before and after incubation with serum. Values are the mean ± S.D. DLC = diamond-like carbon, DLC-PDMS-h = DLC polydimethylsiloxane hybrid, DLC-PTFE-h = DLC polytetrafluoroethylene hybrid, Ta = tantalum, Ti = titanium, Cr = chromium.

5.3.4 Biofilm viability

In the observation of Live/Dead stained biofilm, using CLSM, viable bacteria embedded in the biofilm were seen green and dead bacteria red. Representative samples showed uniform results: the viability of the bacteria was higher in the surface layers of the biofilm, whereas staphylococci in the deeper layers were dead and red (Figure 10). The biofilm landscape formed mushroom-like towers.

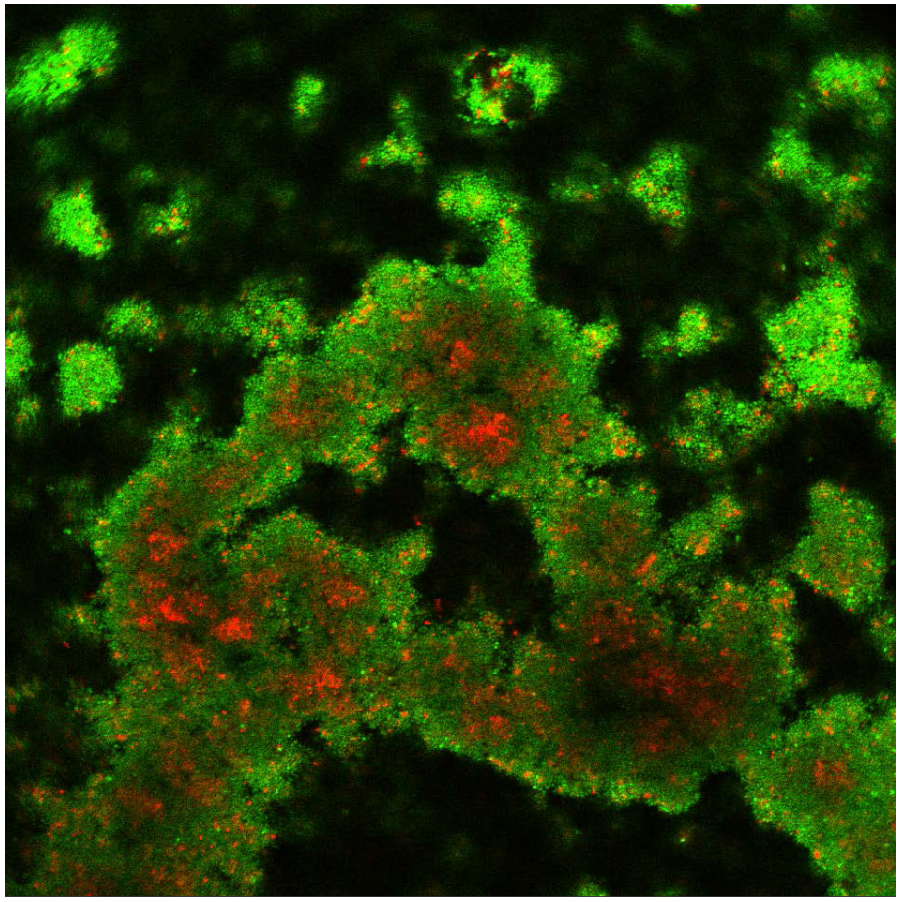


Figure 10. A cross-section of *S.aureus* S-15981 biofilm on a biomaterial surface, viewed with a confocal laser scanning microscope (CLSM) after 48 hours of incubation. In the Live and Dead viability staining, dead bacterial cells are seen in red color and viable cells in green color. The bacteria are embedded in extracellular polymeric substance (EPS).

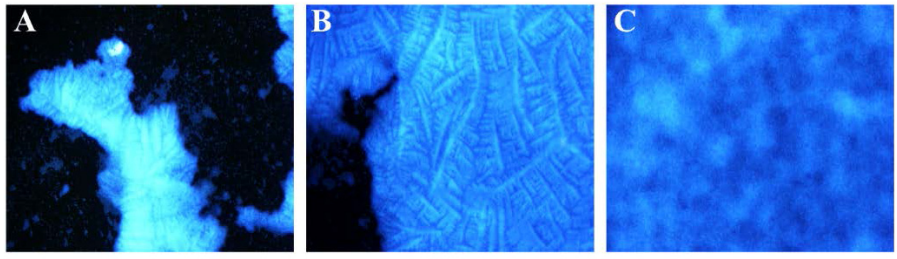


Figure 11. Calcofluor white staining. Calcofluor white stained staphylococcal biofilms after 16 hours of incubation seen on DLC-PTFE-h (A), DLC (B) and titanium (C) biomaterial samples, epifluorescence microscopy. DLC-PTFE-h was clearly the most

resistant to biofilm formation, followed by DLC as second in the ranking order. Titanium surfaces were almost entirely covered by biofilm.

5.4 hBD-3 expression in septic joint implant loosening (Study IV)

5.4.1 Immunohistochemistry

In the peri–implant tissue sections of septic implant loosening, hBD-3 negative, multilobular, polymorphonuclear neutrophils were seen (Table 6.). Circulating monocytes in the vascular lumen were also hBD-3 negative, but some of the monocyte-like cells in tissues were instead hBD-3 positive (Table 6.). Foreign body giant cells were very weakly hBD-3 immunoreactive or hBD-3 negative (Table 6.), whereas macrophage-like (Table 6.) cells were frequently hBD-3 immunoreactive. Some variation could be seen in vascular endothelial cells lining the vascular lumen as some were hBD-3 immunoreactive (Table 6.). Immunoreactivity could be considered in some spindle-shaped stromal fibroblast-like cells localized along wavy and collagenous fibrous tissue (Table 6.). The cellular cytoplasm contained most of the hBD-3 staining while some specific hBD-3 staining could be seen in the ECM in areas infiltrated by hBD-3 immunoreactive macrophage-like cells. The specificity of the extracellular and cellular hBD-3 staining was confirmed with positive sample controls of periodontitis and negative staining controls of peri-implantitis tissues.

The hBD-3 immunoreactive cells were more frequent in women than in men regarding hip joints, whereas contradictory findings could be seen in knee joints. No clear differences in the host hBD-3 response in septic loosening were observed considering the presence or absence of staphylococci. Early revisions (max. 3 months) showed a few hBD-3 immunoreactive cells and foreign body giant cells and substantial numbers of neutrophils. Instead, in peri-implant tissues of implants with longer survival times (up to over 13 years) there could be seen more hBD-3 immunoreactive cells, often macrophage-like cells and additionally some slightly hBD-3 immunoreactive foreign body giant cells.

5.4.2 Double immunofluorescence staining

In double immunofluorescence stainings, CD68 immunoreactive macrophages contained hBD-3. While vascular endothelial cells were mostly hBD-3 negative, in some areas, there could be seen hBD-3 immunoreactive blood vessels, confirmed by double labeling of these cells with hBD-3 and vascular endothelial cell marker CD31 antibodies. No colocalization of hBD-3 and HSP47 proteins was seen in double immunofluorescence stainings. Also, human mast cell tryptase immunoreactive mast cells did not colocalize with hBD-3 positive cells.

Table 6. Cellular sources of hBD-3 in septic joint implant loosening. Periprosthetic tissue samples of septic joint implant loosening were stained for human β-defensin-3 (hBD-3). Observations were made that the circulating blood leukocytes, such as neutrophils, lymphocytes, and monocytes, were hBD-3 negative. The infiltrating macrophages and newly recruited monocytes in tissues were hBD-3 positive. The vascular endothelial cells were usually hBD-3 negative, but in some areas they were hBD-3 immunoreactive, lining the vascular lumen. Fibroblast-like cells were mostly hBD-3 negative, but in some regions, they were hBD-3 positive. The foreign body giant cells were very weakly hBD-3 positive, and the mast cells were hBD-3 negative. + describes that given cells were hBD-3 positive, - refers that given cells were hBD-3 negative, and +/- denotes that both positive and negative cells were found.

Cellular sources of hBD-3 in septic joint implant loosening	
Endothelial cells	+/-
Fibroblasts	+/-
Foreign body giant cells	+/-
Macrophages	+
Mast Cells	-
Monocytes	+/-
Neutrophils	-

6. DISCUSSION

6.1 General discussion

Bacterial adhesion, the first step in the implant infection, was evaluated from multiple viewpoints (Studies I and II). In the third study of this thesis (Study III) biofilm formation following bacterial adhesion was examined. In the context of implant infections, it is relevant to evaluate both phenomena. These infections begin with initial bacterial adhesion but often precede to full-scale biofilm infections that are difficult to diagnose and treat.

The aim was to compare DLC and DLC-p-h to biomaterials traditionally used in orthopedics for their ability to inhibit bacterial adhesion and biofilm formation. Well-established and known methods such as static bacterial cultures, dynamic flow testing, stainings, and microscopy were employed in the experimentation. These methods were modified according to desired purposes.

The experimental setups may result in a considerable variation between different experiments. Hence comparisons of different studies and interpretation of results are challenging. To counter this problem and obtain reliable information about the effect of patterning on bacterial adhesion in the static adhesion tests, we used a different kind of patterned coatings produced on the same background material, silicon. This allows values of various experiments to be normalized. In some experiments, several patterned biomaterial chips were attached to an array plate to bypass natural fluctuations. In this setting, the number of bacteria per macroarray sample was the same for different biomaterials and patterns.

Considering the statistical methods used, the one-way analysis of variance (ANOVA) was used to determine whether there were any statistically significant differences between the means of three or more independent (unrelated) groups. The experiments were run in triplicates from biologically identical bacterial sources. This assured the normality distribution assumption of ANOVA and prevented Type 1 errors to occur. Larger microbial probe and sample numbers may strengthen further the results of adhesion and biofilm studies. However, practical issues in the laboratory work may limit the experimentation possibilities. In this thesis, the use of technical triplicates allowed studies of bacterial adhesion and biofilm formation to be performed with sufficient precision and confidence.

Many aspects of the bacterial adhesion and biofilm formation remains unknown. Comparisons of results between *in vitro* and *in vivo* studies has limitations, due to the uniqueness of the human body as a system. Moreover, the conditioning films established *in vivo* on the implant surfaces are diverse and difficult to mimic. If a given biomaterial is microbe-repellent *in vitro*, it does not straightforwardly mean it will inhibit bacterial adhesion equally well *in vivo*. When investigating the influence of certain surface property on bacterial adhesion and biofilm formation, it is difficult to define confounding factors and to obtain adequate information about them because many properties are interrelated. Considering the concept of competition for the surface between host tissue and bacteria, discussed earlier in this thesis, a co-culture model *in vitro* might better reflect with *in vivo* conditions.

As this thesis concentrates on the medical use of biomaterials in the human implants, serum incubations, and dynamic flow testing were used as methods to create *in vivo* reminiscent conditions. The static bacterial adhesion and biofilm experiments were done in the presence of serum, which significantly affects the binding of bacteria (Patel,JD, et al. 2007). Proteins have sometimes been seen as confounding factors disturbing the "pure" bacterial adhesion; hence buffers such as PBS have been often used as media. However, in the human body, the implant surface is always covered with proteins derived from plasma, and ECM (Francois,P, et al. 1998). At the time of experimentation, serum incubation was a novel method in adhesion and biofilm studies. In the recent published studies, incubation with serum is well-established method in material science.

Both a reference bacterial strain and a clinical isolate were employed as microbiological probes. The use of a reference strain enables reproduction of the experiment by other researchers and comparison to existing studies. These reference strains are laboratory-adapted strains that lose genes due to several passages on the culture medium and can have lower genetic load than clinical strains. A clinical isolate may behave differently and possess different properties compared to the collection strain. The bacterial species tested in this thesis, *S.aureus* or *S.epidermidis,* are pathogens the most frequently implicated in implant infections. However, the taxonomic diversity of different bacterial species and strains is manifested in behavior of different bacteria resulting from unique surface proteins and structures. Therefore, the results of the study may not be valid in all strains and species.

Titanium and tantalum are biomaterials traditionally utilized in orthopedics. Chromium is widely used as a biomaterial component in cobalt-chromium-vanadium and surgical steel alloys but has no use in clinical practice as pure biomaterial due to carcinogenic and allergic properties (Barceloux,DG. 1999). It should be noted, that the results of this study are valid for DLC and DLC polymer hybrid coatings discussed earlier in chapter 2.1.4.2. In the literature, DLC is often used as an "umbrella" term referring to amorphous carbon coatings with "diamond-like" properties, which have many different fractions of diamond sp^3-bonds and varying hydrogen content in the film. The quality of these amorphous carbon coatings is variable and therefore is reported not consistently. The DLC and DLC-p-h coatings produced with the FPAD method and described in this thesis have a high fraction of sp^3 diamond bonds (>80%) (Alakoski,E, et al. 2003, Anttila,A, et al. 1999).

6.2 Bacterial adhesion (Studies I and II)

Both static and dynamic flow conditions were used to investigate bacterial adhesion. Dynamic flow conditions reflect circulation in the human body and the presence of tissue fluids. Common bacterial species were used in testing, and DLC and DLC-p-h were compared to commonly used biomaterials. Quantitative data describing bacterial adhesion was obtained in the experiments.

In the static adhesion study, uniform binding of bacteria to un-patterned coatings was observed. When the biomaterial coating covered 69.4% of the total surface area (when Si

formed the spots on titanium background), the bacteria adhered homogeneously to the surface. Such localization reflects the fact that biofilm formation begins with adhesion of planktonic bacteria evenly to the surface what is typical for bacterial growth from homogenous suspension. If 30.6% of the surface area was covered by bacteria-binding biomaterials, the microbes formed in 90 minutes small biofilm-like bacterial communities. This demonstrates that bacteria divide and earlier start to produce EPS, also called slime. Microorganisms also grew on the silicon background with the smallest patterned spots, only five μm in diameter. The smallest spots are very close to each other, as the total 30.6% covered area by five μm spots is the same as this covered by larger spots. Since the diameter of Staphylococcus is approximately one μm, one can conclude that three staphylococci in a row could bridge the three μm distances between five μm biomaterial spots. One could also think that the EPS matrix might form bridging connections with closest biomaterial spots -"islands." It is likely that these connections form a layer allowing microbes between the spots to avoid a contact with silicon background and make contact with the bacterial slimy layer covering the silicon background.

In dynamic adhesion tests, the contact angles of the tested materials were measured to evaluate the effect of material surface hydrophobicity on bacterial adhesion. Within the conventional biomaterials, tantalum being the most hydrophilic metallic biomaterial had the lowest contact angle, followed by titanium and chromium, which was the most hydrophobic metallic biomaterial. This ranking order was similar to their ability to inhibit bacterial adherence of both *S.aureus* and *S.epidermidis.* This result confirmed the previous published findings that hydrophilicity is connected to the inhibition of bacterial adhesion, whereas hydrophobicity is related to good bacterial adherence to surface of biomaterial.

In case of the DLC and its polymer hybrids DLC-PTFE-h and DLC-PDMS-h there were no associations between hydrophilicity and bacterial adhesion inhibition ability. DLC-PTFE-h and DLC-PDMS-h were the most hydrophobic materials with equally high contact angles, but DLC-PTFE-h inhibited bacterial adhesion in dynamic conditions best of all tested materials. Dynamic adhesion results show that hydrophobicity is not the only determining factor in the bacterial adhesion process.

It could be hypothesized that the relationship between adherence and hydrophobicity might follow the same pattern as has been shown for the relationship between adherence of bone marrow-derived mesenchymal stromal cells and hydrophobicity. Stromal cells adhere better to materials with an intermediate contact angle than to more hydrophilic or hydrophobic surfaces. The turning point of this parabolic relationship for these cells is around the contact angle 60°(Shin,YN, et al. 2008). But this parabolic relationshipaxiom can not explain why DLC–PTFE-h had the lowest *S. aureus* binding ability whereas the DLC–PDMS-h surface with the same contact angle (104°) bind the highest number of *S. aureus* cells. Observations made in the dynamic testing agree with the conclusions made by Raulio *et al*. and Jones *et al.* (Jones,DS, et al. 2006, Raulio,M, et al. 2008).

The measured sliding angles of DLC, DLC-PTFE-h, and DLC-PDMS-h did not correlate with their contact angles in dynamic adhesion study. It was seen that both DLC polymer hybrids had the same contact angles, but their sliding angles were different. Similar findings were reported by Kiuru and Alakoski (Kiuru,M and Alakoski,E. 2004) and Chen *et al.* (Chen,W, et al. 1999).

Surface roughness is biomaterial surface property often evaluated in bacterial adhesion and biofilm studies. After thin film deposition the surface roughness values of the samples in the static *S.aureus* experiments were below 10 nm. In the dynamic adhesion study, the surface roughness values of the samples were in a very narrow range between 2 and 25 nm. According to the previous findings (Bollen,CM, et al. 1996), material surface roughness values in these size ranges do not play any critical role in results of the experiments. The observed differences in surface roughness do not contribute to confounding factor nor offer any explanation for the test results. Significant R_a values affecting biofilm retention would be in the range of 200 nm or higher (Bollen,CM, et al. 1996, Quirynen,M, et al. 1996)

The results of static adhesion testing differ from those obtained under dynamic conditions. Flow conditions affect behavior and surface structures of bacteria and this may explain these differences. The static experiments were done in the presence of serum, which signficantly affects the binding of bacteria (Patel,JD, et al. 2007). Adhesion of bacteria *in vivo* is very complex phenomenon and optimal model has not been yet found. Presently, no single straightforward explanation for our adhesion results could be proposed. The outcome might be related to the sp^3, and sp^2 ratio in DLC material coatings or other bacteria-biomaterial interaction property such as zeta potential or other yet unknown factors. The sp^3 and sp^2 hybridized carbon-carbon bond ratios in DLC materials affect their properties (Lifshitz,Y. 1999), which might have an influence on the experimental results. Sp^3 and sp^2 bond ratios in our tested new DLC-p-h materials are not very well known yet.

6.3 Biofilm formation (Study III)

Serum incubations were used in the biofilm study to create *in vivo* reminiscent conditions. Common bacterial species and biomaterials currently applied in clinics were chosen for testing. This study not only examined the ability of the biomaterial surface to inhibit biofilm formation, but also the retention of biofilm on the surface and the ability of the surface to allow detachment of biofilm. During the experiment, when biofilm covered samples were washed with 0.9% NaCl, an unknown amount of bacteria and biofilm detached from the surface, as an air-liquid interface was formed. An air-liquid interface associated with bacterial biofilm assays (Gomez-Suarez,C, et al. 2001), and the dynamic equilibrium between the forces of biofilm retention and removal (Quirynen,M and Bollen,CM. 1995), are evaluated in the literature.

It has been thought that high-energy surfaces bind bacteria and allow biofilm formation more than low-energy surfaces (Quirynen,M and Bollen,CM. 1995, Teughels,W, et al. 2006) and hydrophobicity has been associated with inhibition of bacterial adhesion (Roosjen,A, et al. 2006, Tang,H, et al. 2009). Though controversial data exists showing that hydrophobic surfaces allow more adhesion (Cerca,N, et al. 2005, MacKintosh,EE, et al. 2006, Patel,JD, et al. 2007). Both conclusions might be true, as is suggested by Baier (Baier,RE. 2006). Baier has studied the relationship between surface free energy and particulate or bacterial adhesion and proposed a curve as an explanation. He concluded

that there is an optimal value (around 25 mJ/m^2) of surface free energy for minimal bacterial adhesion (Baier,RE and DePalma,VA. 1971). According to parabolic curve, surface free energy values under and above the optimal value give enhanced bacterial adherence. This paradigm is described as Baier's curve (Baier,RE and DePalma,VA. 1971, Baier,RE. 1980, Baier,RE, et al. 1984, Pereni,CI, et al. 2006). This has relevance to the results of Study III, where it was found that the best material to inhibit biofilm formation was DLC-PTFE-h with a surface free energy of 24.5 mJ/m^2. If only hydrophobicity of the material surface were considered, one would think that the low surface free energy and high contact angle materials DLC-PTFE-h and DLC-PDMS-h would be the best, but as described before, bacterial adhesion and biofilm formation are multifactorial processes, and other factors also may play a role.

The zeta potential values of all materials were in a narrow range of -43.6 mV to -54 mV after serum incubation, indicating repulsive interactions between the negatively charged material surfaces and the bacterial slime. For the two best biofilm formation repelling materials, DLC PTFE-h and DLC the zeta-potential values measured before serum incubation and the lowest recorded, were -84.2 mV and -88.4 mV respectively. After serum incubation, they were less negative and closer to the level of the values of the other materials analysed in this study. An increase from -84.2 mV to -48.8 mV was observed for DLC-PTFE-h and from -88.4 mV to -52.3 mV for DLC. Other materials showed smaller changes in their zeta potential values. It could be suggested that the more noticeable change of zeta potential values of DLC-PTFE-h and DLC surfaces might be associated with their different charge and an amount of the proteins coating on the DLC-PTFE-h and DLC surfaces as compared with other materials.

In serum, proteins differ extensively in their charge and electrokinetic properties. Despite similar zeta-potential values after serum incubation, it is likely that the conditionings films on materials differ from each other in the type and conformational changes of the adsorbed proteins (Buchanan,LA and El-Ghannam,A. 2010). The size and quantity of the protein film, as well as the quality and conformation of adsorbed proteins, vary on different surfaces. The conformation of adsorbed proteins and ligands exposed to the body and bacteria are influenced by the material surface chemistry characteristics, as has been examined before (Baugh,L and Vogel,V. 2004, Keselowsky,BG, et al. 2003). Biomaterial surface chemistry has long-term effects on biofilm formation in the presence of serum proteins, as seen in the work done by Patel et al. with *S.epidermidis* (Patel,JD, et al. 2007).

Two biomaterial properties, low surface energy and low zeta-potential affecting the conditioning protein film may explain inhibition of biofilm formation and poor retention of biofilm on DLC and DLC-PTFE-h. As these two materials were clearly the best in the study, it seems that the zeta-potential of the surface may play the main role because changes of DLC and DLC-PTFE-h electrokinetic potentials were different from changes of DLC-PDMS-h and other materials in the study. The difference between the two best-performing biomaterials in the biofilm study might relate to hydrophobicities of the surfaces as DLC-PTFE-h surface is more hydrophobic than DLC surface (water contact angles 106^0 and 67.4^0, respectively). The conditioning film derived from incubation with serum affects biofilm formation, as it changes the zeta potential and contact angle values of the tested biomaterials. It has been reported that cell-to-cell binding and cell-to-matrix

adhesion is even more important for the biofilm formation than binding through physicochemical attraction forces (Cerca,N, et al. 2005, MacKintosh,EE, et al. 2006). Biofilm formation is thus affected not only by chemical and physical characteristics of the biomaterial surface but also by the adhesion molecules on the bacterial surface and the serum-derived counter-ligands on the biomaterial surface (Cerca,N, et al. 2005). Regarding surface roughness values of the tested biomaterials in the biofilm study, the samples had R_a values of equal or less than 2 nm and a smooth mirror quality comparable to the mirror finish surfaces of medical implants.

Recently, it has even been shown that super hydrophobic and super hydrophilic surfaces can both decrease biofilm formation. As an example, a lotus leaf has a water contact angle of 170º, low surface energy and specific surface roughness with microprotrusions and nano hairs with excellent self-cleaning properties (Zhang,X, et al. 2013). The development of super hydrophobic (Zhang,X, et al. 2013) and super hydrophilic (Mi,L and Jiang,S. 2014) materials has been gaining more interest in material sciences.

One can find in the literature many different explanations of the bacterial adhesion results and biofilm studies. The single factors or material/surface properties such as hydrophobicity, topography, surface charge, roughness, and chemistry have been examined for years. Super-hydrophobic surfaces, super-hydrophilic surfaces, negatively charged surfaces and nm-scale surface roughness have all been shown to decrease bacterial adhesion. However, as bacterial adhesion and biofilm formation have become better known as complex phenomena, it is the most likely that several factors and properties might play a role. The results of the studies in this thesis lead to similar conclusions. This can also be confirmed by of *in vitro* studies performed with different experimental settings showing contradictory results (Cerca,N, et al. 2005, Patel,JD, et al. 2007, Roosjen,A, et al. 2006, Tang,H, et al. 2009).

Live/Dead staining of biofilm was used to visualize bacteria clustered in the slimy EPS matrix; especially the viability of the bacteria was screened. Live/Dead stainings showed that the viable (stained green) bacteria were located in the proximity of the biofilm surface while the dead (stained red) bacteria were situated in the interior and bottom of the mushroom-like biofilm towers. The explanation for this could be that nutrients and oxygen diffuse with difficulties to the deeper sectors of the biofilm. The bacteria located deep in the biofilm might die of starvation because the top surface localized microbes might use much of the nutrients. Patel *et al.* also viewed the biofilm after Live/Dead staining and reported similar findings (Patel,JD, et al. 2007)

6.4 hBD-3 in septic implant loosening (Study IV)

The cellular sources of origin of hBD-3 in septic joint implant loosening were described. According to past studies, hBD-3 can be found in the synovial fluid in septic implant loosening. The concentrations of hBD-3 were shown to be even higher in synovial fluid than in serum (Gollwitzer,H, et al. 2013), suggesting local production and secretion of hBD-3 in peri-implant tissues in septic loosening. An increased mean optical density of

hBD-3 was observed in periprosthetic tissues of septic loosening patients compared with aseptic loosening patients, the spacer group, and normal controls in hBD-3 immunostaining (Liu,GD, et al. 2014). This indicates that the intensity of hBD-3 staining of positive cells or the proportion of the hBD-3 immunoreactive cells was increased, what indicates a local synthesis and release of hBD-3 in septic loosening. The results of this thesis explain the increased tissue and synovial fluid concentrations of hBD-3 in septic implant loosening.

The heterogeneous and local expression of hBD-3 in peri-implant tissues in septic implant loosening is consistent with the findings showing that the hBD-3 is absent in healthy synovial tissues. The hBD-3 has been found in synovial membranes of osteoarthritis, psoriatic arthritis (Paulsen,F, et al. 2002) and rheumatoid arthritis (Bokarewa,MI, et al. 2003) patients without any clear links to an infection. Since hBD-3 was not found in healthy synovial membranes (Paulsen,F, et al. 2002) the upregulation of hBD-3 may be inflammation-related. Instead, hBD-1 is present in the healthy synovial membrane. Moreover, hBD-3 may also describes the severity of the infection and inflammation in periprosthetic tissues in PJIs (Gollwitzer,H, et al. 2013, Liu,GD, et al. 2014).

Less sensitive indirect immunofluorescence method may not show minor immunoreactive hBD-3 accumulations related to fibroblasts and foreign body giant cells. This is in agreement with the reports that the modern multilayer methods such as peroxidase-anti-peroxidase complex (and avidin-biotin-peroxidase complex method) (Moran,MM, et al. 1985) have been shown to be 100 -to 1,000-fold more effective than indirect immunofluorescence in immunodetection. These modern methods allow 100 -to 1,000-fold higher dilutions of primary antibodies (Beckett,JH and Bigbee,JW. 1979). The horseradish peroxidase labeled polymer method used in the study of this thesis also is two to fivefold more sensitive compared to multilayer methods mentioned above (Sabattini,E, et al. 1998). Nevertheless, a minor role for fibroblast as a potential cellular source of hBD-3 in synovial fluid and peri-implant tissues in septic loosening is suggested, but remains controversial (Dunsche,A, et al. 2002). The very low amount of hBD-3 seen in labeled polymer stained fibroblasts might relate to exogenous hBD-3 bound (Nishimura,M, et al. 2004) or taken up (Semple,F and Dorin,JR. 2012) by fibroblasts. Neutrophils and mast cells, cells equipped with storage granules, were hBD-3 negative in this current study.

Regulation of hBD-3 in septic loosening was further analyzed in different sub-groups. The analysis suggested that the expression of hBD-3 is differently regulated in women and men. It is likely that this difference is more related to differences in the anatomy and implant loading than sex steroid effects on host response, considering that the study group have older patients. The presence or absence of staphylococci did not relate to the host hBD-3 response. This might be due to hBD-3 regulation by a variety of PAMPs and tissue derived DAMPs. The histology of the peri-implant tissues was associated with the survival time of the implants, which might be related to mechanisms of tissue pathology.

In septic loosening the locally produced hBD-3 probably functions as a site- and time-specific effective anti-microbial agent, host defense molecule (Dhople,V, et al. 2006, Wang,G. 2014). It has efficacy against staphylococci, the micro-organisms most often implicated in PJIs (Arciola,CR, et al. 2012, Montanaro,L, et al. 2011) and even against MRSA. Additionally, hBD-3 has activity against Gram-negative bacteria and fungi

(Feng,Z, et al. 2005, Harder,J, et al. 2001). hBD-3 has thus broader antimicrobial capacity in contrast to hBD-1 and hBD-2, which are active against Gram-negative bacteria and some fungi. Considering that hBD-3 binds broadly to several microbes, it might offer potential in medical imaging and as a diagnostic asset in the detection of septic implant loosening (Leung,K. 2009).

Regarding limitations of the current study of this thesis, the sample number was low. This means that the subgroup analysis by age, gender, the location of the implant, type of the pathogen and the survival time of the implant are not definite and need to be examined further with larger patient and sample series. Secondly, hBD-3 was localized by using only immunostaining. Other methods would have strengthened the demonstration of hBD-3 in different cell types in septic loosening. Nevertheless, the specificity of the immunostaining was confirmed by sample and staining controls.

6.5 Clinical implications and future perspectives

The complexity of the bacterial interactions, the ability of bacteria to persist in the extreme environments and antibiotic resistance, are main challenges in the prevention of implant infections. The high degree of genomic diversity among bacterial strains and species and the difficulties of translating animal models to human trials have hindered the progress in therapeutical solutions. In this thesis, an approach for fighting infections is provided in the form of coatings that could minimize bacterial adhesion and biofilm formation on a biomaterial surface.

DLC and its new polymer hybrids DLC-PTFE-h and DLC-PDMS-h were tested against conventional biomaterials used in medical implants regarding their ability to inhibit bacterial adhesion and biofilm formation. The DLC inhibited bacterial adhesion in static conditions and biofilm formation better than conventional implant materials. It was as good as conventional implant materials in dynamic flow testing of bacterial adhesion. The novel and anti-soiling DLC-PTFE-h was as good as conventional implant biomaterials when bacterial adhesion was tested in flow conditions, but showed the highest resistance to biofilm formation.

According to the current studies, DLC and DLC-PTFE-h are microbe-repellent biomaterials and could be utilized to combat implant infections. Due to its mechanical properties such as hardness and wear resistance, DLC could be applied as a coating for orthopedic implants. DLC-PTFE-h could be utilized in medical devices, e.g. guidewires, cardiac catheters and artificial heart valves to prevent implant-related infections. The obtained results could not be explained solely by the measured biomaterial physicochemical surface properties.

The cellular sources of origin of hBD-3 antimicrobial peptide were identified in peri-implant tissues in septic implant loosening. Given the unique role of hBD-3 as a powerful natural antibiotic against even multiresistant (Maisetta,G, et al. 2006) and naturally tolerant (Gottlieb,CT, et al. 2008) bacterial strains, it seems that the pharmaceutical field will explore more the possible utilization of hBD-3. As the broad use of antibiotics is

associated with resistance and adverse effects, alternative treatment strategies such as the hBD-3 should be studied further.

In future study experimentation efforts should be made to mimic *in vivo* conditions even more extensively, and *in vivo* animal experiments would be essential to obtain information relevant to clinical practice. The diversity of the microorganisms warrants multiple pathogens as testing probes and using wild-type clinical isolates are of great importance. When the effect of biomaterial surface properties on bacterial adhesion and biofilm formation is assessed, several parameters should be taken into consideration. In the future, the emphasis in the research efforts should be laid on the competitions between bacteria and tissue cells for the free implant surface. In such studies, simultaneous growth of bacteria and tissue cells on the same biomaterial surface is evaluated.

7. CONCLUSIONS

Study I. Under static conditions and in the presence of serum proteins, the DLC was more microbe-repellent than conventional biomaterials. Micropatterned surfaces are useful for quantitative evaluation of bacterial adhesion on biomaterials. Different sized microtextures enable data collection regarding bacterial alignment and colonization on surfaces.

Study II. The DLC and DLC-PTFE-h were as good as conventional biomaterials in their ability to inhibit bacterial adhesion under dynamic flow conditions. The DLC-PDMS bound significantly more *S.aureus* than DLC-PTFE-h and tantalum.

Study III. The DLC and DLC-PTFE-h were more resistant to biofilm formation than conventional biomaterials and DLC-PDMS-h in the presence of serum. The DLC-PDMS-h was as good as conventional biomaterials in its ability to inhibit biofilm formation. The DLC-PTFE-h excelled the anti-biofilm ranking..

Study IV. The cellular sources of the antimicrobial peptide hBD-3 in peri-implant tissues in septic implant loosening are mainly macrophages and vascular endothelial cells. The hBD-3 was not found in neutrophils or mast cells. The heterogeneous topological expression of hBD-3 in peri-implant tissues in septic implant loosening designates local induction, which is well in line with earlier findings in the literature.

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